Simultaneous stressors: interactive effects of an immune challenge and dietary toxin can be detrimental to honeybees

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Abstract

Recent large-scale mortality of honeybee colonies is believed to be caused by multiple interactions between diseases, parasites, pesticide exposure, and other stress factors. To test whether a dual challenge has an additive effect in reducing survival, we experimentally stimulated the immune system of caged *Apis mellifera scutellata* workers from six colonies by injecting saline or *E. coli* lipopolysaccharides (LPS), and additionally fed them the alkaloid nicotine (0 µM, 3 µM and 300 µM in 0.63 M sucrose). Workers did not increase their sucrose intake to compensate for the immune system activation, and those injected with *E. coli* LPS decreased their intake on the highest nicotine concentration. In the single challenges, injection and high nicotine doses negatively affected survival. All injected worker groups showed reduced survival. Without nicotine, survival of the saline and *E. coli* LPS worker groups was similar, but survival of *E. coli* LPS-challenged workers dropped below that of the saline groups when additionally challenged by nicotine, with bees dying earlier at higher nicotine concentrations. In the dual challenge of saline injection and dietary nicotine, a reduced effect on survival was observed, with lower mortality than expected from the summed mortalities due to the single challenges. However, additive and synergistic effects on survival were observed in workers simultaneously challenged by *E. coli* LPS and nicotine, indicating that interactive effects of simultaneous pathogen exposure and dietary toxin are detrimental to honeybee fitness.

Keywords: *Apis mellifera scutellata*; *E. coli* lipopolysaccharide; nicotine; sugar intake; survival

1. Introduction

Current pollinator declines are of great concern around the globe (Potts et al., 2010), because an estimated 87.5% of flowering plants rely on animal pollinators for reproduction (Ollerton et al., 2011). Researching the causes of pollinator declines is essential, as losses of food products and biodiversity are immediate threats (Biesmeijer et al., 2006; Gallai et al., 2009). Honeybees provide substantial pollination services to agricultural crops and wild plants (Aebi et al., 2012; Ellis, 2012; Morse, 1991), and major colony losses in the recent past are of great concern (vanEngelsdorp et al., 2009, 2011, 2012). Multiple stressors may contribute to this population decline: bacterial and viral diseases (Forsgren, 2010; Genersch, 2010; Tentcheva et al., 2004), energetic stress and immune system suppression caused by the parasitic mite *Varroa destructor* (Amdam et al., 2004; Yang and Cox-Foster, 2005), insecticide exposure, and nutrient deficiencies (Brodschneider and Crailsheim, 2010; Mullin et al., 2010; Naug, 2009; Williams et al., 2010).

Large-scale honeybee colony losses are likely to be caused by integrative effects of multiple stressors (Aebi and Neumann, 2011; Dainat et al., 2012a; Maini et al., 2010; Potts et al., 2010), and recent studies have begun to examine the impact of different factors acting in concert. Simultaneous insecticide exposure and *Nosema* infection had a negative synergistic effect on honeybee immunity and survival, meaning that the negative effect of the dual challenge was greater than the additive effect of the two challenges individually (Alaux et al., 2010a; Aufauvre et al., 2012). Pettis et al. (2012) demonstrated that *Nosema*-infected workers had an increased spore count when they originated from colonies exposed to sub-lethal insecticide doses over several weeks, compared to infected workers from control colonies. Another study, involving analysis of dead workers collected from colonies, showed that lifespan of winter bees was

reduced by *V. destructor* and deformed wing virus, while infections with *Nosema ceranae* or acute bee paralysis virus were not linked to survival (Dainat et al., 2012b).

We are interested in whether the additive effect of an immune challenge and dietary toxin is more detrimental to honeybees than either challenge alone. Insect haemolymph contains pattern recognition proteins that bind to surface molecules of microbes and induce immune responses, including phenoloxidase activation and antimicrobial peptide production (Kanost et al., 2004). An attack by bacteria can be simulated by exposing the insect to nonviable and non-pathogenic bacterial surface molecules, namely lipopolysaccharides (LPS) (Azumi et al., 1991; Hultmark, 1993; Kato et al., 1994). LPS activate the Jun N-terminal Kinase (JNK) pathway of the innate immune system, increasing expression levels of the immune response gene Defensin2 in the fat bodies of honeybees (Richard et al., 2008). In adult workers, rupturing the cuticle by saline injection has been found to cause an immune response, but *Escherichia coli* LPS injection causes a much stronger increase in Defensin2 expression (Richard et al., 2008). A recent study confirmed that antimicrobial peptide synthesis increased following an injection with either Ringer or LPS, and young workers further had a lower phenoloxidase activity after LPS injection (Laughton et al., 2011). The immune response is costly: bumblebees (*Bombus terrestris*) can tolerate an *E. coli* LPS challenge when food is available *ad libitum*, but when additionally challenged by starvation they show poorer survival than starved control individuals (Moret and Schmid-Hempel, 2000).

In addition to activation of the immune system, we challenged honeybees by including nicotine in their diet. Nicotine is a naturally occurring and highly toxic alkaloid (Detzel and Wink, 1993; Kessler et al., 2010; Yildiz, 2004), that functions as a defence against herbivores (Steppuhn et al., 2004). Nicotine is also used as insecticide in organic farming (Casanova et al.,

2002; Isman, 2006), although its use declined after introduction of its synthetic analogues (neonicotinoids) (Matsuda et al., 2001; Tomizawa and Casida, 2005). Nicotine and neonicotinoids have the same action at the nicotinic acetylcholine receptors (Tomizawa and Casida, 2005). Naturally occurring nectar nicotine concentrations (≤30 µM) do not have a negative effect on honeybees, but ingestion of higher nicotine doses (300 µM) reduces the survival of larvae and workers (Köhler et al., 2012; Singaravelan et al., 2006).

In the present study, caged honeybee worker groups were exposed to an immune challenge (by injecting saline or *E. coli* LPS) and fed nicotine-containing diets. Different worker groups were exposed to either one of the two challenges, while others experienced the dual challenge of immune system activation and dietary toxin. We predicted that food intake would not differ between control and nicotine diets, but that injected workers would have a higher energy intake than untreated individuals to compensate for the immune challenge. In the single challenges, worker survival was expected to be reduced on the high nicotine concentration, and also following *E. coli* LPS injection. In the dual challenges, we expected to find an additive, or even synergistic, effect on longevity, with workers exposed to *E. coli* LPS and high nicotine concentration showing the lowest survival.

2. Materials and methods

2.1. Experimental procedure

Frames with capped worker brood were removed from six *Apis mellifera scutellata* colonies at the University of Pretoria Experimental Farm. Experiments were carried out in winter and spring (July to September 2011). Workers were collected within 24 h of emergence (N=900 per colony) and randomly assigned to one of three treatments (total N=1800 per treatment; Fig. 1): 1) *No*

injection: workers that were not injected, i.e. a full control; 2) *Saline*: injection of 3 µl phosphate buffered saline; and 3) *E. coli LPS*: injection of 3 µl 0.5 mg/ml *E. coli* lipopolysaccharides (strain O128:B12; Ref. L2755, Sigma Aldrich, Munich, Germany). The *E. coli* LPS concentration was chosen based on an earlier study, where the injection of 4 µl 0.5 mg/ml *E. coli* LPS into 7-day old *A. m. carnica* workers did not cause mortality within 8 h (Richard et al., 2008). Bees were injected in the abdominal cavity, between the 3rd and 4th tergite, using a Hamilton microsyringe. No anaesthesia was required as newly emerged workers do not sting or fly. Workers were caged in groups of 100 individuals, resulting in a total of 54 cages. Cages (11×8.5×7 cm) contained a piece of honeycomb (5×5 cm) and were incubated at 34 ± 1˚C and 45% relative humidity in darkness, to mimic conditions within the hive. Two plastic feeding vials (15 ml) with a cut feeding hole (1×0.3 cm) were inserted horizontally into the cages, one with water and one with the experimental diet, both provided fresh daily.

The three cages of each treatment and each colony were randomly assigned to one of three experimental diets (Fig. 1), consisting of a 0.63 M sucrose solution with 0 µM, 3 µM and 300 µM (–)-nicotine (Ref. N3876, Sigma Aldrich). Protein was not included in the diet, as caged *A. m. scutellata* survive longest on sugar-only diets (Pirk et al., 2010). Experiments lasted for 21 days, with food intake being recorded daily by weighing the feeders (±0.1 mg, Mettler Toledo AG-64, Microsep Ltd, Johannesburg, South Africa). Survival was assessed by counting and removing dead bees daily.

2.2. Data analysis

Food intake was corrected for evaporation (determined from feeders in empty cages), and daily sucrose intake per bee was calculated to correct for decreasing group size over time. Analysis

was performed on summed intake of the first seven days, when the majority of workers was alive in all cages. Intake data were tested for normality (Kolmogorov-Smirnov) and homogeneity of variance (Levene's test), and were compared between treatments (*no injection*, *saline*, *E. coli LPS*) and diets (0, 3, 300 µM nicotine) using one-way and two-way ANOVA. *Post-hoc* comparisons were conducted with Tukey's HSD test. Kaplan-Meier survival regression analyses were performed to test for differences in survival between treatments and diets, followed by Gehan's Wilcoxon pair-wise comparisons. Observed mortality of workers exposed to the dual challenge was compared to the expected mortality (calculated as sum of the mortalities of injected workers and uninjected ones fed nicotine) using χ^2 tests. Statistical analysis was done in Statistica^{TM} V. 10. Level of significance was $\alpha<0.05$; data are presented as means ± SE.

3. Results

3.1. Effect of immune challenge and nicotine on sucrose intake

Sucrose intake did not differ between the treatments ($F_{2,45}$=0.68, P=0.51), but differed between the nicotine concentrations ($F_{2,45}$=5.62, P<0.01). There was no significant interaction between treatment and diet ($F_{2,45}$=0.85, P=0.50). When looking at the treatments separately, sucrose intake of *no injection* workers summed over the first seven days did not differ between the nicotine concentrations ($F_{2,15}$=0.28, P=0.76), neither did sucrose intake of *saline* workers ($F_{2,15}$=2.67, P=0.10; Fig. 2). However, sucrose intake of *E. coli LPS* workers was found to differ between diets ($F_{2,15}$=3.81, P=0.05), being lower on the 300 µM nicotine diet than on the no nicotine and 3 µM nicotine diets (P=0.05; Fig. 2).

3.2. Effect of immune challenge and nicotine on worker survival

Workers from the *no injection* groups survived significantly better than either *saline* or *E. coli LPS* workers, irrespective of dietary nicotine ($Z \geq 13.59$, $P<0.001$; Fig. 3). On average, 26 ± 2% of the workers died within 24 h of being injected. Survival of *saline* and *E. coli LPS* workers was similar on the no nicotine diet ($Z=0.11$, $P=0.91$), but survival of *E. coli LPS* workers was poorer than that of *saline* workers on both nicotine diets ($Z \geq 3.24$, $P<0.01$; Fig. 3). On the 3 µM nicotine diet, survival of *saline* and *E. coli LPS* workers was similar in the first seven days, after which survival of *E. coli LPS* workers fell below that of *saline* workers. On the high nicotine concentration (300 µM), survival of *E. coli LPS* workers was lower than that of *saline* workers from Day 2 onwards (Fig. 3). Comparing worker longevity between the three diets within a treatment, it was found that survival of *no injection* workers differed between the nicotine concentrations ($\chi^2=55.57$, $P<0.001$), with workers living longer on the no nicotine and 3 µM nicotine diets than on 300 µM nicotine ($Z \geq 4.02$, $P<0.001$). Survival of *E. coli LPS* workers also differed between the diets ($\chi^2=38.62$, $P<0.001$), but these workers showed lower survival on both nicotine diets than on the no nicotine diet ($Z \geq 4.23$, $P<0.001$). Interestingly, survival of *saline* workers did not differ significantly between diets ($\chi^2=4.79$, $P=0.09$).

As can be seen in Table 1, mortality was more than twice as high in *E. coli LPS* and *saline* workers as in *no injection* workers when they were fed the highest nicotine concentration. Thus, a single injection had a stronger effect on survival than chronic ingestion of the nectar alkaloid. In the dual challenge of saline injection and dietary nicotine, the interactive effect on worker survival was lower than expected from the added mortalities of the two individual challenges (Table 1). For example, saline injection reduced survival by 54.7% compared to *no injection*, and 300 µM nicotine reduced survival by 20.6%. An additive effect of both challenges

combined would give a decrease in survival of 75.3%, but a reduced effect on survival was observed ($\chi^2 \geq 95.23$, df=5, $P<0.001$; Table 1), with the mortality of workers exposed to the dual challenge being similar to that due to the saline injection alone. In workers simultaneously challenged by *E. coli LPS* and nicotine, on the other hand, an additive effect on longevity was observed on the 300 µM nicotine diet ($\chi^2=0.00$, df=5, $P=1.00$). On the low nicotine concentration, mortality was even higher than predicted from an additive effect, i.e. a synergistic effect was observed ($\chi^2=1256.74$, df=5, $P<0.001$; Table 1).

4. Discussion

We have provided experimental evidence of an immune challenge and chronic toxin ingestion interactively affecting honeybee longevity. Workers that were both injected with *E. coli* LPS and fed nicotine showed the highest mortality. Looking at the challenges individually, the once-off immune system activation with either saline or *E. coli* LPS had a larger effect on honeybee survival than the ingestion of the nectar alkaloid nicotine throughout the experiment. Below we discuss behavioural and physiological consequences of the challenges; and possible fitness effects of multiple stressors acting in concert.

4.1. Effects of the immune challenge

We observed a high mortality rate of *saline* and *E. coli LPS* workers within 24 h after injection. The stress of handling and injecting and the wound itself may have caused the deaths of the young workers. Mortality of honeybees following injection with control buffers was similarly high (20% after 48 h) in an earlier study (Picard-Nizou et al., 1997) and has been attributed to the

trauma associated with injection. Many studies do not report the mortality of injected honeybees and bumblebees and just work with the survivors. Mealworms (*Tenebrio molitor*) and starved bumblebees also showed reduced survival following injection with non-infectious substances (Moret and Schmid-Hempel, 2000; Pursall and Rolff, 2011). Handling of insects during injection induces a stress response and the resulting tissue damage further poses a risk of secondary infection (Kucharski and Maleszka, 2003). The behaviour of honeybees is also altered following handling stress: workers handled without anaesthesia showed lower sucrose responsiveness 30 min after handling than anaesthetized individuals (Pankiw and Page, 2003). *E. coli* LPS-injected honeybees were further found to have a lower ability to associate an odour with a food reward than control bees, suggesting that the immune response interferes with learning or memory formation (Mallon et al., 2003). A delayed onset of feeding of injected workers in our experiment may have contributed to the reduced weekly sucrose intake (Fig. 2), and the energy deficit may have increased mortality.

An immune response has significant energetic costs in insects (DiAngelo et al., 2009; Freitak et al., 2003), and is likely to result in a compensatory increase in food intake. However, honeybees in our study did not increase their food intake following saline or *E. coli* LPS injections. A recent study also found no difference in cumulative sucrose consumption over three weeks between workers challenged sequentially or simultaneously with *Nosema ceranae* and fipronil at different ages (Aufauvre et al., 2012). Contrary to this, honeybees infected with *N. apis* and *N. ceranae* consumed more sucrose than uninfected individuals (Alaux et al., 2010a), and bumblebees increased their energy intake when their immune system was activated with LPS injections (Tyler et al., 2006). While individuals in these two studies were fed pollen in addition to sugar solution, workers in our study were not provided with protein, which is likely to affect

the immune response. Pathogen resistance and immune function of insects is improved when their diet is supplemented with protein (Alaux et al., 2010b; DeGrandi-Hoffman et al., 2010; Lee et al., 2006 and 2008). Following an immune challenge, caterpillars select higher dietary protein to carbohydrate ratios (Lee et al., 2006; Povey et al., 2009). Future studies should investigate whether honeybees live longer after injection and LPS exposure when protein is included in their diet, and whether they choose protein-rich diets following an immune challenge.

4.2. Effects of dietary nicotine

Uninjected workers tolerated even the high nicotine concentration and defended their sucrose intake on all diets. This confirms our earlier finding in caged workers, despite free-flying foragers being repelled by 300 µM nicotine when given a choice between multiple feeders containing 0 – 1000 µM of the alkaloid in 0.63 M sucrose (Köhler et al., 2012). Honeybees can taste bitter substances in nectar (Wright et al., 2010), and post-ingestive detection of nicotine has also been shown in insects (Glendinning, 1996). Despite being able to detect the alkaloid, uninjected honeybee workers maintained their nectar intake under no-choice conditions, thus defending a constant energy intake.

In the *no injection* groups, the low nicotine concentration did not affect worker longevity, while 300 µM nicotine reduced survival, which is in agreement with our earlier study examining the effect of dietary nicotine on worker survival (Köhler et al., 2012). Nicotine affects various biological functions through its action on acetylcholine receptors in the nervous system (Kleinsasser et al., 2005; Thany and Gauthier, 2005; Yildiz, 2004). In insects, nicotine affects sucrose perception and olfactory memory (Thany and Gauthier, 2005), reduces nutrient assimilation efficiency (Bentz and Barbosa, 1990), and its detoxification imposes metabolic and

fitness costs (Cresswell et al., 1992). Digestive interference and additional energetic costs may have contributed to the increased mortality of our honeybee workers. Negative effects of nicotine on insect performance and survival have been shown in multiple studies (Harvey et al., 2007; Krug and Proksch, 1993; Singaravelan et al., 2006).

4.3. Interactive effects of immune challenge and dietary nicotine

Nicotine ingestion affected survival of *saline* and *E. coli LPS* worker groups differently. *Saline* workers maintained their food intake on the nicotine diets, as did untreated controls. However, while uninjected workers showed reduced survival on the highest nicotine concentration, survival of *saline* workers did not differ between diets. Contrary to our prediction, the interactive effect of *saline* and nicotine ingestion was lower than expected from the mortality caused by either challenge individually, i.e. a positive synergistic effect on survival was observed. Negative effects of nicotine ingestion may have been offset by its antimicrobial properties, lowering the risk of infection at the injection site. Nicotine has been shown to inhibit the growth of various bacterial and fungal pathogens (Pavia et al., 2000), and also inhibits viral replication (Yamashina et al., 2008). *Manduca sexta* caterpillars infected with *Bacillus thuringiensis* survived better when nicotine was included in their diet (Krischik et al., 1988). We noticed in our earlier study that honeybee workers from certain colonies showed lower survival on sucrose-only diets compared to the majority of workers, and these potentially diseased honeybees survived better when nicotine was added to their diet, possibly as a result of antimicrobial benefits (Köhler et al., 2012).

In contrast to *saline* workers, *E. coli LPS* workers reduced their food intake on the highest nicotine concentration, thus ingesting less toxin but also less energy. These workers

showed reduced survival on both nicotine concentrations, compared to the no nicotine diet. Nicotine is known to affect adaptive and innate immune responses in mammals, inhibiting the antibody-forming cell response, T-cell proliferation and fever response (Kalra et al., 2004; Razani-Boroujerdi et al., 2011). In mice injected with live *E. coli*, nicotine ingestion suppresses the immune response and inflammation, leading to increased bacterial counts in tissues and reduced survival (van Westerloo et al., 2005). Mammalian studies further show that activating the cholinergic anti-inflammatory pathway with nicotine inhibits LPS-induced excessive inflammation and prevents long-term physiological and behavioural distortions, thus accelerating recovery from the sick state (Kojima et al., 2011; Wittebole et al., 2007). To our knowledge, no studies to date have simultaneously exposed insects to LPS and nicotine. In contrast to the findings in mammals, we did not find evidence for nicotine being beneficial to *E. coli* LPS-challenged honeybees. As predicted from earlier studies on simultaneous stressors, we found additive, and even synergistic, effects on worker survival, with workers challenged by *E. coli* LPS and nicotine having the shortest lifespan.

Comparing *saline* and *E. coli LPS* worker survival, no difference was found when the honeybees were fed the no nicotine diet. However, when they were also exposed to nicotine, survival of *E. coli LPS* workers dropped below that of *saline* workers. Similarly, bumblebees treated with LPS and fed *ad libitum* survived as well as individuals injected with Ringer, but when additionally stressed by starvation, they showed poorer survival than the starved controls (Moret and Schmid-Hempel, 2000). Nicotine also enhances negative effects in insects stressed by parasitism, compared to non-parasitized individuals (Bentz and Barbosa, 1990; Gunasena et al., 1990). As an example of a dual challenge in honeybees, the interaction between insecticide

exposure and *Nosema* infection weakened bees much more than individual challenges (Alaux et al., 2010a; Aufauvre et al., 2012; Pettis et al., 2012).

Our study adds to the scant but urgently needed evidence for interactive effects of simultaneous stressors on pollinator fitness (Potts et al., 2010). We found that simultaneous exposure to *E. coli* LPS and nicotine reduces honeybee longevity as much as, or more than, expected from an additive effect of the two challenges individually. An immune challenge and toxin exposure acting in concert can potentially weaken honeybee colonies, while the cost of either challenge alone may go unnoticed if honeybees are not exposed to further stressors. There is clearly a need to investigate the interactive effects of multiple stressors, such as parasites, diseases, pesticides and dietary quality, on honeybee colony fitness. A better understanding of multi-challenge consequences for fitness parameters like foraging behaviour, survival and reproductive success is crucial to explain and mitigate population declines of honeybees and other pollinators.

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Table 1. Day-to-day survival of *A. m. scutellata* workers subjected to saline or *E. coli* LPS injection, and/or fed one of two nicotine concentrations (3 µM = low N; 300 µM = high N) in 0.63 M sucrose for 21 days. Day-to-day survival is expressed as percentage difference from the absolute control (*no injection* + no nicotine diet), averaged over the 21-day experiment (N=600 workers from six colonies per diet per treatment; mean ± SE). Observed survival on the dual challenges was compared to survival expected from an additive effect (sum of mortalities on the two individual challenges) using χ^2 tests.

Challenge	Observed survival (% difference to control)	Expected survival (additive effect)	Obs. *vs*. exp.	Observed effect
Saline	– 54.7 ± 4.9			
E. coli LPS	– 49.2 ± 2.7			
low N	– 7.9 ± 2.5			
high N	– 20.6 ± 3.6			
Saline + low N	– 55.8 ± 3.2	– 62.6	***	Synergistic (positive)
Saline + high N	– 55.4 ± 4.4	– 75.3	***	Synergistic (positive)
E. coli LPS + low N	– 70.9 ± 5.1	– 57.1	***	Synergistic (negative)
E. coli LPS + high N	– 65.8 ± 4.8	– 69.8	n.s.	Additive

Figure legends

Figure 1. Experimental design. *Apis mellifera scutellata* workers from six colonies were subjected to three treatments (*no injection*; *saline*; *E. coli* lipopolysaccharides), and were fed one of three experimental diets differing in nicotine content.

Figure 2. Sucrose intake of *A. m. scutellata* workers over the first seven days of the experiment (N=600 workers from six colonies per diet per treatment; mean + SE). Workers were subjected to three treatments (*no injection*; *saline*; *E. coli LPS*), and were fed one of three experimental diets (0.63 M sucrose with 0 µM, 3 µM and 300 µM nicotine). *E. coli LPS* workers decreased their sucrose intake on the high nicotine concentration (Tukey HSD following one-way ANOVA: **P*=0.05).

Figure 3. Cumulative survival of *A. m. scutellata* workers over the 21-day experiment (N=600 workers from six colonies per diet per treatment; mean ± SE; SE partly omitted for clarity). Workers were subjected to three treatments (*no injection*; *saline*; *E. coli LPS*), and fed one of three experimental diets (0.63 M sucrose with 0 µM (top), 3 µM (middle) and 300 µM (bottom) nicotine). Statistical differences between data series derive from Gehan's Wilcoxon pair-wise comparisons and are indicated by different letters.

Figures

Figure 1.

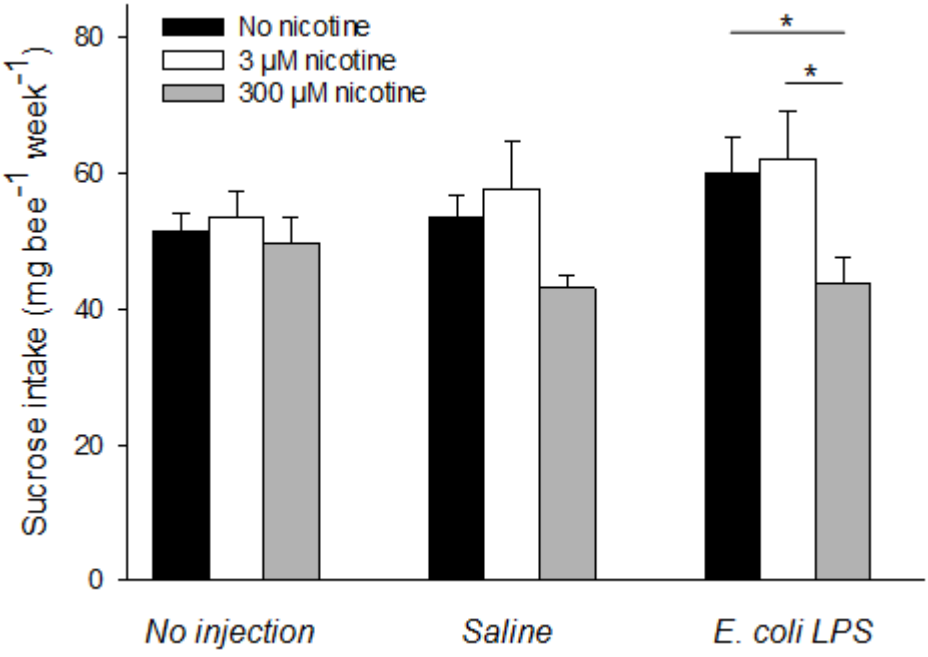
No nicotine
3 µM nicotine
300 µM nicotine
Sucrose intake (mg bee-1 week-1)
80
60
40
20
0
*
*
No injection
Saline
E. coli LPS

Figure 2.

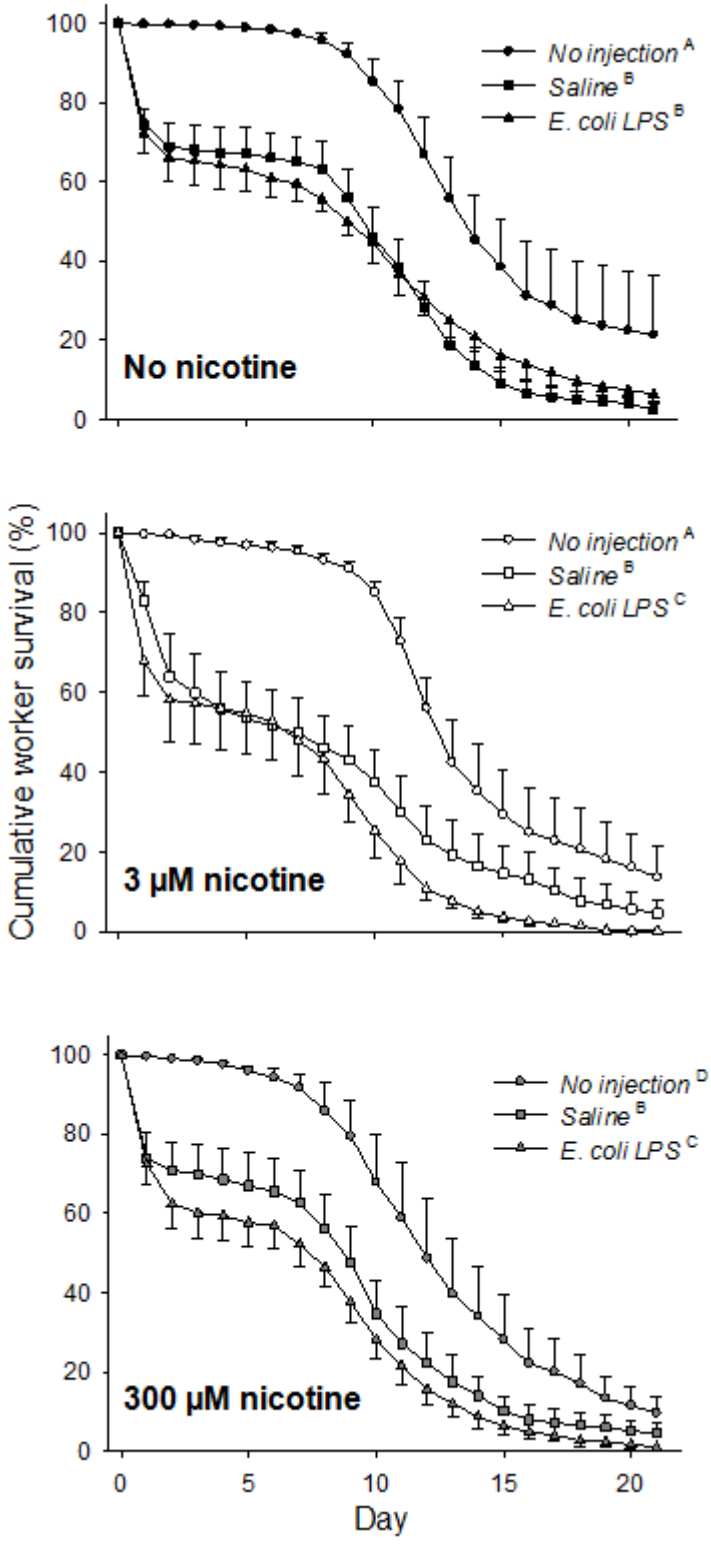
Cumulative worker survival (%)
100
80
60
40
20
0
No injection A
Saline B
E. coli LPS B
No nicotine
No injection A
Saline B
E. coli LPS C
3 µM nicotine
No injection D
Saline B
E. coli LPS C
300 µM nicotine
0
5
10
15
20
Day

Figure 3.