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Acute exposure to selenium disrupts associative conditioning and long-term memory recall in honey bees (*Apis mellifera*)

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ABSTRACT

A plethora of toxic compounds – including pesticides, heavy metals, and metalloids – have been detected in honey bees (*Apis mellifera*) and their colonies. One such compound is selenium, which bees are exposed to by consuming nectar and pollen from flowers grown in contaminated areas. Though selenium is lethal at high concentrations, sublethal exposure may also impair honey bees' ability to function normally. Examining the effect of selenium exposure on learning and memory provides a sensitive assay with which to identify sublethal effects on honey bee health and behavior. To determine whether sublethal selenium exposure causes learning and memory deficits, we used proboscis extension reflex conditioning coupled with recall tests 30 min and 24 h post-conditioning. We exposed forager honey bees to a single sublethal dose of selenium, and 3 h later we used an olfactory conditioning assay to train the bees to discriminate between one odor associated with sucrose-reinforcement and a second unreinforced odor. Following conditioning we tested short- and long-term recall of the task. Acute exposure to as little as 1.8 ng of an inorganic form of selenium (sodium selenate) before conditioning caused a reduction in behavioral performance during conditioning. And, exposure to 18 ng of either an inorganic form (sodium selenate) or an organic form (methylseleno-L-cysteine) of selenium caused a reduction in the bees' performance during the long-term recall test. These concentrations of selenium are lower than those found in the nectar of plants grown in selenium-contaminated soil, indicating that even low-grade selenium toxicity produces significant learning and memory impairments. This may reduce foragers' ability to effectively gather resources for the colony or nurse bees' ability to care for and maintain a healthy colony.

1. Introduction

With the global decline in honey bee (*Apis mellifera*) populations, there is heightened interest in the factors that influence their survival. In addition to the normal challenges of predators and natural environmental dynamics – such as weather and resource availability – honey bees face a multitude of human-generated factors – such as toxin release into the environment – that negatively affect their health. In order to understand how to better manage our honey bee populations in the face of these human-generated factors, we need to know the effects of each individual toxin on honey bee health and behavior at sublethal, as well as lethal, levels.

One of the challenges honey bees are currently facing is the accumulation of naturally occurring toxic chemicals, such as selenium, in the environment. In addition to being released into the environment by the natural weathering of rocks, selenium is released in larger quantities from metal ore during metal extraction, from coal and petroleum during burning, and from phosphate containing rocks that are used to manufacture agricultural fertilizer (Lakin, 1972). High soil concentrations of selenium have been found in areas contaminated with runoff from heavily used agricultural areas, industrial waste sites, and mining waste dumps (Mehdi et al., 2013). Selenium contamination from agricultural runoff is widespread across the western United States, affecting approximately 1.5 million acres across 8 states (Brown, Jr. et al., 1999). In areas contaminated with toxic levels of selenium, plants can accumulate high levels of selenium in nectar and pollen, which is then collected by foraging pollinators, like honey bees, and fed to the young in the colony (El Mehdawi and Pilon-Smits, 2012; Hladun et al., 2013, 2012; Quinn et al., 2011).

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In vertebrates, trace amounts of selenium are known to be essential for normal development, antioxidant protein and enzyme function, and hormone regulation (Letavayova et al., 2006). These essential functions are mainly mediated through its participation in antioxidant activities when selenium is incorporated into selenoproteins. However, when ingested at high concentrations, selenium becomes toxic. Excess selenium catalyzes the production of reactive oxygen species, causing oxidative damage, that can result in developmental abnormalities, neurological impairment, and death (Letavayova et al., 2006). In comparison little is known about the requirement and functions of selenium and the mechanisms of selenium toxicity in invertebrates.

In adult honey bees, a single dose of selenium greater than 60 mg/L causes a significant increase in mortality within 5 days of exposure (Hladun et al., 2012). Yet, honey bees do not appear to be able to taste the presence of even lethal concentrations of selenium in a sucrose solution with antennal or proboscis stimulation (Hladun et al., 2012). They therefore readily consume the highly contaminated food. Foragers will also bring the contaminated nectar and pollen back to the hive, which exposes the rest of the bees to toxic levels of selenium.

Even sublethal concentrations may have a significant effect on honey bee health and behavior. The accumulation of sublethal concentrations of selenium in the hive may impair forager bees' ability to efficiently gather resources for the colony and nurse bees' ability to maintain the hive and care for the brood and the queen. This would further compromise colony health even before selenium accumulates to a lethal concentration and increase the colony's susceptibility to other toxins, disease, or infestation by pests or parasites. However, the effect of sublethal selenium exposure on honey bee behavior is still largely unknown.

In this study, we used a discrimination conditioning paradigm and memory tests coupled with acute sublethal selenium exposure to explore the possibility of selenium induced impairments in honey bee behavior. Hladun, et al. (2012) described a reduction in some honey bee feeding behaviors and survival following consumption of selenium contaminated food (Hladun et al., 2013, 2012). However these assays are not sensitive enough to resolve some of the more subtle behavioral effects sublethal selenium exposure, such as learning and memory impairments. Conditioning the proboscis extension reflex (PER) in honey bees is a more sensitive measure for the influence of toxic compounds on neural function and behavior (Smith and Burden, 2014). PER learning and memory tests can also provide information about potential mechanisms for how sublethal selenium toxicity influences honey bee behavior.

We hypothesized that acute exposure to sublethal levels of selenium would reduce honey bees' performance during conditioning and the recall tests. The impaired performance would likely be seen as a reduced proportion of bees responding to the rewarded odor, especially during the long-term recall test. This would indicate impairments in the ability or the motivation to respond to olfactory stimuli, or it may be attributed to a disruption of processes required for effective learning and memory consolidation.

2. Material and methods

2.1. Animals and selenium exposure

For this study, bees were collected from 3 colonies with open-mated New World Carniolan queens. Returning foragers were captured at the entrance of the hive in the morning. Only bees not carrying a pollen load were collected. The bees were briefly cold anesthetized and restrained in custom harnesses that left their proboscis and antennae free to move normally. After they recovered from the anesthetization, the bees were divided into treatment groups.

In a first set of experiments, the bees were then fed 3 µl of either 0.5 M sucrose solution or 0.5 M sucrose + selenium 3 h prior to beginning conditioning. All bees were able to consume the whole dose of selenium-contaminated sucrose. The two selenium compounds used were sodium selenate (BioXtra, Sigma-Aldrich, Saint Louis, MO) and methylseleno-L-cysteine, 98% (Acros Organics, Pittsburgh, PA). Sodium selenate and methylseleno-L-cysteine are the predominant forms found in many flower parts, including nectar and pollen, of several plant species (Hladun et al., 2013; Quinn et al., 2011). The concentrations of both selenium compounds used in this study were 0.6mg/L (1.8 ng), 6 mg/L (18 ng), or 60 mg/L (180 ng). These concentrations were shown to be sublethal following a single acute exposure and are comparable to and lower than the ranges of selenium concentrations found in nectar of plants grown in selenium-contaminated greenhouse or natural environments (Hladun et al., 2013, 2012).

In a second set of experiments, the bees were fed 3 µl 0.5 M sucrose without selenium before conditioning. They were then fed 3 µl of either 0.5 M sucrose or 6 mg/L selenium in 0.5 M sucrose 3 h before the beginning of a long-term recall test. For this second set of experiments, the 6 mg/L concentration was chosen for the selenium treatment group since it had the greatest effect on honey bee behavior during the first set of experiments.

Following dosing, the animals were left undisturbed in a humidified plastic box for 3-4 h. Next, just prior to the beginning of olfactory conditioning, we performed a motivation test in which each bee was tested for proboscis extension reflex (PER) to antennal stimulation with a droplet of 1.5 M sucrose, which they were not allowed to consume. This test provided a measure of the reduction in motivation to feed following selenium ingestion. And, only bees that showed PER to sucrose stimulation were used in olfactory conditioning, as they were sufficiently motivated to learn the task.

2.2. Odor stimulation

The two odors used for olfactory conditioning and test trials were 2M 1-hexanol (Sigma-Aldrich, St. Louis, MO) and 2M 2-octanone (Fluka, Sigma-Aldrich, St. Louis, MO). These odors have been used in several previous experiments investigating odor perception and olfactory learning in honey bees (Thorn and Smith, 1997; Wright et al., 2009, 2005). Odors were diluted in heavy mineral oil (Sigma-Aldrich, St. Louis, MO).

Odor cartridges consisted of a glass 1 cc tuberculin syringe barrel (BD Medical, Franklin Lakes, NJ) with a short length of silicon tubing (Cole-Parmer, VernonHills, IL) as a constriction in the broad end. 10 µl of an odor solution was placed on a small strip of filter paper (Whatman 114, Sigma-Aldrich, St. Louis, MO) inside each odor cartridge. The odor cartridge was connected to the automated odor delivery system via tubing attached to the narrow end of the cartridge and placed so the broad end was approximately 2 cm from the bee's antennae when she was in the conditioning arena.

The odors were presented via an automated odor delivery system coordinated by a DirectLogic 05 programmable logic controller (Automation-Direct, Cumming, GA) that triggers the opening of a valve (The Lee Co., Westbrook, CT), re-directing an airstream (~400 ml/min) through the odor cartridge. During odor stimulation, the airstream was passed through the odor cartridge, pushing odor-laden air toward the bee's antennae. A continuous flow exhaust system, located approximately 5 cm behind the bee, removed the odor from the conditioning area after every trial to maintain temporally discrete odor exposure.

2.3. Olfactory conditioning

The animals were conditioned to discriminate between the two odors. Each bee was exposed to the two odors in a pseudorandomized sequence of 16 trials (+ − − + − + + − + − − + − + + −) or (− + + − + − − + − + + − + − − +), where '+' represents the sucrose-reinforced odor (CS+) and '−' represents the unreinforced odor (CS−) (Smith and Burden, 2014; Smith et al., 1991). The odor used as the CS+ was alternated with each repetition of the experiment. The conditioning paradigm allowed us to assess the effect of selenium exposure on each bee's ability to discriminate the two odors, in addition to assessing the acquisition of the conditioned associations.

For each trial the bee was placed within the conditioning area and allowed to acclimate for a few seconds. During presentation of the odor stimulus the airstream was directed through the odor cartridge for 4 seconds. On CS+ trials, the odor stimulus was forward-paired with 0.6 µl 1.5 M sucrose. The sucrose was delivered 3 s after odor onset to allow for a 1 s overlap between the odor stimulus and the reward. On CS− trials, the odor stimulus was not paired with any reward. At the end of each type of trial the bee was left undisturbed in the conditioning area for a few seconds before she was removed and placed into a holding area. The inter-trial interval was 8 minutes.

Individual responses to each conditioning trial during the acquisition phase were recorded as binary yes/no responses. A positive response to the odor stimulus was defined as the presence of the proboscis extension reflex (PER) during the olfactory stimulus and before presentation of sucrose for the CS+ trials (Smith and Burden, 2014). PER was defined as the extension of the proboscis beyond an imaginary line drawn between the tips of the opened mandibles. The overall percentage of bees exhibiting PER to any given conditioning or recall test trial (% PER) was calculated and used as an overall measure of the bees' performance during conditioning and recall testing.

2.4. Short-term and long-term recall test trials

Approximately 30 min following the end of the acquisition phase, each bee was exposed to a single unreinforced test trial with each odor. The presence or absence of PER was once again recorded as a binary variable. Following the short-term recall test trials, the bees were fed to satiation with 0.5 M sucrose and placed in a humidified box overnight. Then, 24 h later, the bees were exposed to a series of 3 unreinforced test trials of each odor presented in the same pseudorandomized sequence used during conditioning. The odor presented first during both short- and long-term recall test trials was alternated with each daily repetition of the experiment. The odor presented first during conditioning was presented second during the test trials.

Immediately following the short-term recall test trials and again following the long-term recall test trials, we stimulated the bees' antennae with a small droplet of 1.5 M sucrose. The presence or absence of PER in response to the stimulation was recorded as a binary yes/no variable. This sucrose responsiveness test was a measure of how motivated the bees were to feed and thus to respond to the olfactory stimulus. It also allowed us to assess whether the motor/feeding responses were affected by selenium.

2.5. Graphing and statistical analysis

The proportion of bees responding during the preconditioning motivation test and the presence/absence scoring for the conditioning trials, short-term recall test trials, and long-term recall test trials were plotted as the percentage of bees exhibiting PER to each trial (% PER).

All statistical analyses were performed in SPSS Statistics v.22. We used a Pearson's Chi-square test to determine if there was a significant difference between treatment groups in the number of bees exhibiting PER during the sucrose responsiveness tests before conditioning and following the short- and long-term recall test trials. The differences across treatment groups in the probability of the bees exhibiting PER during odor stimulation were analyzed using logistic regression via generalized estimating equations (Logistic GEEs) with Least Squares Difference *post hoc* pairwise comparisons. We assessed the bees' performance during conditioning, the short-term recall test, and the long-term recall test separately using models including main effects and all appropriate 2-way and 3-way interaction terms. When interaction terms were not significant they were removed from the model. The final reduced models are reported. Predictors for the models included: (1) trial number (TRIAL), (2) the square of each trial number ($TRIAL^2$) to account for nonlinear increase in % PER over the trials, (3) the difference between responses to the sucrose-reinforced odor and the unreinforced odor during the experiments (ODOR), and (4) the concentration of selenium the bees were exposed to (DOSE). ODOR and DOSE were entered into the models as categorical predictors. Possible correlations between the repeated measurements taken from individual bees were accounted for by a within-subject variable identifying the responses by each bee (BEEID). This variable does not appear in the models below as it was an internal parameter used to adjust the significance levels for each of the predictors mentioned above.

3. Results

3.1. Sodium selenate

In the experiment where the bees were treated with sodium selenate prior to conditioning, we tested the bees' sucrose responsiveness just before beginning conditioning to determine whether selenium altered their motivation to feed. Approximately 90–95% of the bees in the control group and each treatment group responded during this preconditioning sucrose responsiveness test (Table S1). There was no significant effect of treatment with sodium selenate on the number of bees that responded with PER during the sucrose responsiveness test (Pearson's Chi-square: $\chi^2 = 1.603$, $df = 3$, $p = 0.659$).

Bees in the control group and all treatment groups were able to learn the task and discriminate between the CS+ and the CS− during conditioning (Fig. 1A, Table 1). Over the conditioning trials, the percentage of bees responding to the CS+ increased in all groups. And, there was a significant effect of ODOR on the probability of exhibiting PER, with the percentage of bees responding to the CS+ being significantly higher than the percentage responding to the CS− trials.

All of the selenate treated groups showed a reduction, relative to control, in their responses to the CS+ (Fig. 1A, Table 1). Only the 0.6 mg/L and 6 mg/L sodium selenate treated groups showed a significantly lower percentage of bees exhibiting PER to the CS+ compared to the control group. Although the group treated with 60 mg/L showed a lower percentage of bees responding to the CS+ compared to controls, this decrease was not significant. There was not a significant difference between the percentages of bees responding to the CS− across the treatment groups.

Recall tests also showed differences between control and selenium treated groups. However, these differences were significant only for the long-term recall tests. During the short-term recall test, the bees in all groups discriminated between the CS+ and CS− (Fig. 1A, Table 1). As in acquisition, the lowest response was in the group treated with 6 mg/L selenium. However, the

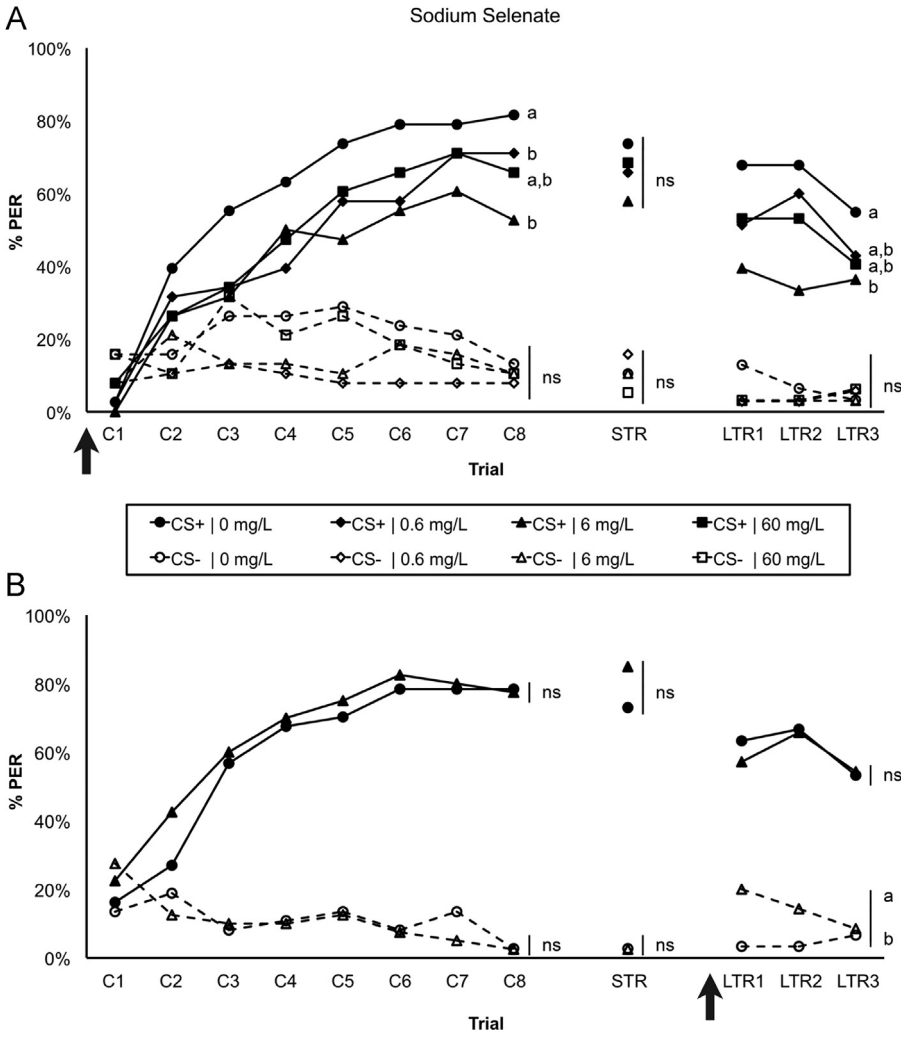

Fig. 1. The percentage of sodium selenate treated bees exhibiting PER (% PER) to each trial during acquisition trials (C1-C8), test trials immediately following conditioning (STR), and test trials approximately 24 h following conditioning (LTR1-LTR3). Bees were either treated with sodium selenate 3 h prior to conditioning (A; for C1–C8 & STR: $n=37$–38, for LTR1–LTR3: $n=31$–35) or 3 h prior to the long-term test trials (B; for C1–C8 & STR: $n=35$–36, for LTR1–LTR3: $n=30$–35). CS+ indicates the sucrose-reinforced odor, while CS− represents the unreinforced odor. The arrows indicate the timing of selenium treatment.

difference in the percentage of bees exhibiting PER to unreinforced trials of the CS+ between treatment groups and the controls failed to reach significance for any comparison. There were also no significant differences in the percentages of bees responding to the

Table 1
Logistic Generalized Estimating Equations analysis of the bees' responses during conditioning, short-term recall, and long-term recall of bees treated with sodium selenate 3 h before conditioning. The bolded p-values are those significantly correlated with the probability of bees exhibiting PER to the conditioning or test trials.

		Conditioning		**Short-term Recall**		**Long-term Recall**	
Predictor	**Contrast**	β^1		β^1		β^1	
		Wald χ^2	***p-Value***	***Wald χ^2***	***p-Value***	***Wald χ^2***	***p-Value***
ODOR	CS− vs. CS+	1.75		6.401		3.110	
		218	***0.000***	*31.038*	***0.000***	*132.437*	***0.000***
DOSE	0 mg/L vs. 0.6 mg/L	−0.718		0.326		−0.531	
		6.43	***0.011***	*0.235*	*0.628*	*1.855*	*0.173*
DOSE	0 mg/L vs. 6 mg/L	−0.738		0.262		−1.106	
		4.86	***0.027***	*0.138*	*0.710*	*6.404*	***0.011***
DOSE	0 mg/L vs. 60 mg/L	−0.401		−0.560		−0.604	
		1.64	*0.200*	*0.598*	*0.439*	*2.460*	*0.117*
TRIAL		0.941		–––		0.423	
		101	***0.000***			*0.616*	*0.433*
TRIAL^2		−0.075		–––		−0.150	
		70.8	***0.000***			*1.272*	*0.259*

[1] The parameter estimate indicates the relationship of the predictor to the percentage of bees responding during conditioning and recall.

Table 2
The responses to sucrose responsiveness tests following the short- and long-term recall tests. Reported are the numbers of bees exhibiting PER to the sucrose stimulation and, in parentheses, the total number of bees in each treatment group.

	Short-term recall				Long-term recall			
Selenium exposure	**0 mg/L**	**0.6 mg/L**	**6 mg/L**	**60 mg/L**	**0 mg/L**	**0.6 mg/L**	**6 mg/L**	**60 mg/L**
Sodium selenate before conditioning	37 (*38*)	38 (*38*)	37 (*38*)	38 (*38*)	28 (*31*)	32 (*35*)	27 (*33*)	29 (*32*)
Sodium selenate before long-term recall	35*(*37*)	–––	36*(*40*)	–––	27 (*30*)	–––	33 (*35*)	–––
Methylseleno-L-cysteine before conditioning	30 (*30*)	28 (*29*)	31 (*31*)	30 (*30*)	24 (*29*)	25 (*28*)	26 (*30*)	24 (*28*)
Methylseleno-L-cysteine before long-term recall	25 (*26*)	–––	30 (*30*)	–––	24 (*26*)	–––	26 (*30*)	–––

* We are missing data from several bees for these values. All bees that were tested showed positive sucrose responsiveness.

CS− across the treatment groups.

During the long-term recall test trials, the bees also discriminated between the CS+ and the CS− (Fig. 1A, Table 1). There was no significant effect of TRIAL on the probability of exhibiting PER, which indicates that there was little detectable extinction as a result of the unreinforced trials with the CS+. In the group treated with 6 mg/L sodium selenate a significantly lower percentage of bees exhibited PER to the CS+ during the long-term recall test trials. The groups treated with 0.6 mg/L and 60 mg/L sodium selenate exhibited smaller decreases in the percentage of bees responding to the CS+, although these decreases were not significant. There were no significant differences between the percentages of bees responding to the CS− across the treatment groups.

Following the short- and long-term recall trials, we tested the bees' motivation to feed and motor function by antennal stimulation with 1.5 M sucrose. The number of bees responding to stimulation was a measure of the bees' motivation to feed and to respond to a feeding cue. Their ability to respond to the stimulation by extension of the proboscis was also an assessment of motor function. There was no difference in the percentage responding to the sucrose stimulation across all control and treatment groups for bees treated with sodium selenate (Table 2; Pearson's Chi-square: short-term recall $\chi^2=2.027$, $df=3$, $p=0.567$; long-term recall $\chi^2=3.264$, $df=3$, $p=0.353$).

We trained a second set of bees using the same discrimination conditioning paradigm. However, instead of selenium exposure prior to conditioning, we exposed the treated group of bees to 6 mg/L sodium selenate 3 h before the beginning of the long-term recall test.

Both the control group and the selenium-treated group increased their response to the CS+ over the course of conditioning and exhibited significant discrimination between the CS+ and the CS− (Fig. 1B, Table 3). There was no significant difference between the performance of the control and treated bees during conditioning, which is expected since they did not differ in terms of treatment during this phase.

We then tested short-term recall as before. Both groups discriminated well between the CS+ and CS− during the short-term recall test (Fig. 1B, Table 3). There was no significant difference between the performance of bees in the control group and treatment group during the short-term recall test, as expected since the groups had been treated identically up to that point.

Following treatment with sodium selenate, we tested long-term recall. Both groups discriminated between the CS+ and CS− during the long-term recall test (Fig. 1B, Table 3). There was no effect of TRIAL or TRIAL^2 on the percentage of bees exhibiting PER, indicating no significant extinction of the conditioned response. There was a significant effect of DOSE and the DOSE × ODOR interaction, indicating that bees treated with sodium selenate showed a significantly greater percentage responding to the CS− than the control bees, though there was no significant difference between the control and treatment groups' responses to the CS+.

After the short- and long-term recall trials, we tested the bees' motivation and motor function by antennal stimulation with 1.5 M sucrose. For the short-term recall sucrose responsiveness test, all of the bees tested for sucrose responsiveness exhibited PER to the sucrose stimulation, so the Pearson's Chi-square statistic could not be calculated. As before, there was no difference in the percentage responding to the sucrose stimulation following long-term recall trials between the control group and sodium selenate treated group. (Table 2; Pearson's Chi-square: long-term recall $\chi^2=0.369$, $df=1$, $p=0.544$).

Table 3
Logistic Generalized Estimating Equations analysis of the responses during conditioning, short-term recall, and long-term recall of bees treated with sodium selenate 3 h prior to beginning the long-term recall test. The bolded *p*-values are those significantly correlated with the probability of bees exhibiting PER to the conditioning or test trials.

		Conditioning		Short-term Recall		Long-term Recall	
Predictor	**Contrast**	β^1		β^1		β^1	
		***Wald* χ^2**	***p-Value***	***Wald* χ^2**	***p-Value***	***Wald* χ^2**	***p-Value***
ODOR	CS− vs. CS+	2.65		5.06		3.58	
		131	***0.000***	*39.1*	***0.000***	*38.9*	***0.000***
DOSE	0 mg/L vs. 6 mg/L	0.155		0.603		1.31	
		0.275	*0.600*	*1.39*	*0.238*	*4.90*	***0.027***
TRIAL		0.694		–––		0.682	
		16.4	***0.000***			*0.685*	*0.408*
TRIAL^2		−0.056		–––		−0.212	
		13.3	***0.000***			*1.09*	*0.297*
ODOR × DOSE		–––		–––		−1.41	
						4.48	***0.034***

[1] The parameter estimate indicating the relationship of the predictor to the percentage of bees responding during conditioning and recall.

3.2. Methylseleno-L-cysteine

Prior to conditioning, we tested the bees' sucrose responsiveness to determine their motivation to feed. Approximately 95–98% of the bees in each treatment group responded during this preconditioning sucrose responsiveness test (Table S1). There was no significant effect of treatment with methylseleno-L-cysteine on the number of bees that responded with PER to the sucrose responsiveness test (Pearson's Chi-square: $\chi^2=1.001$, d$f=3$, $p=0.801$).

For all groups, the percentage of bees responding to the CS+ increased over the conditioning trials and the bees successfully discriminated between the CS+ and CS−, indicating the bees in all of the groups learned the task (Fig. 2A, Table 4). Unlike bees treated with sodium selenate, there was no significant effect of methylseleno-L-cysteine on the percentage of bees exhibiting PER to the CS+ or the CS− during conditioning.

Following conditioning, we also performed recall tests. In the short-term recall test, the bees discriminated between the CS+ and CS−, but there was no significant effect of methylseleno-L-cysteine treatment on the percentage of bees responding to the CS+ and CS- (Fig. 2A, Table 4). During the long-term recall test, all control and treatment groups exhibited discrimination between the CS+ and the CS–. However, in contrast to the short-term tests, there was a significant decrease in the percentage of bees treated with methylseleno-L-cysteine responding to CS+ and to CS−. The group treated with 6 mg/L methylseleno-L-cysteine exhibited a significantly lower % PER to the CS+ test trials than the control group. Over the 3 long-term recall trials, there was a significant decline in the percentage of bees responding to the trials, indicating a significant extinction of the conditioned response overall. Additionally, for the 6 mg/L treatment group, the interaction terms DOSE × TRIAL and DOSE × TRIAL^2 were significant. This interaction shows a significant reduction in the percentage of these bees responding to successive long-term recall trials and a further reduction in the responses to the first and third long-term recall trials in relation to the second trial, compared to the opposite trend seen in control bees.

Following the short-term and long-term recall tests we determined the bees' response levels to antennal stimulation with sucrose. There was no difference in the percentage responding to the sucrose stimulation across all treatment groups for bees treated with methylseleno-L-cysteine (Table 2; Pearson's Chi-square, short-term recall: $\chi^2=3.164$, d$f=3$, $p=0.367$; long-term recall: $\chi^2=1.017$, d$f=3$, $p=0.797$).

We exposed different groups of bees to methylseleno-L-cysteine (6 mg/L) 3 h before the beginning of the long-term recall test (Fig. 2B, Table 5). During conditioning the bees were divided into two equally sized groups. Both groups showed increased percentage of responding to the CS+ over conditioning trials and discriminated between the CS+ and CS− odors. There was no

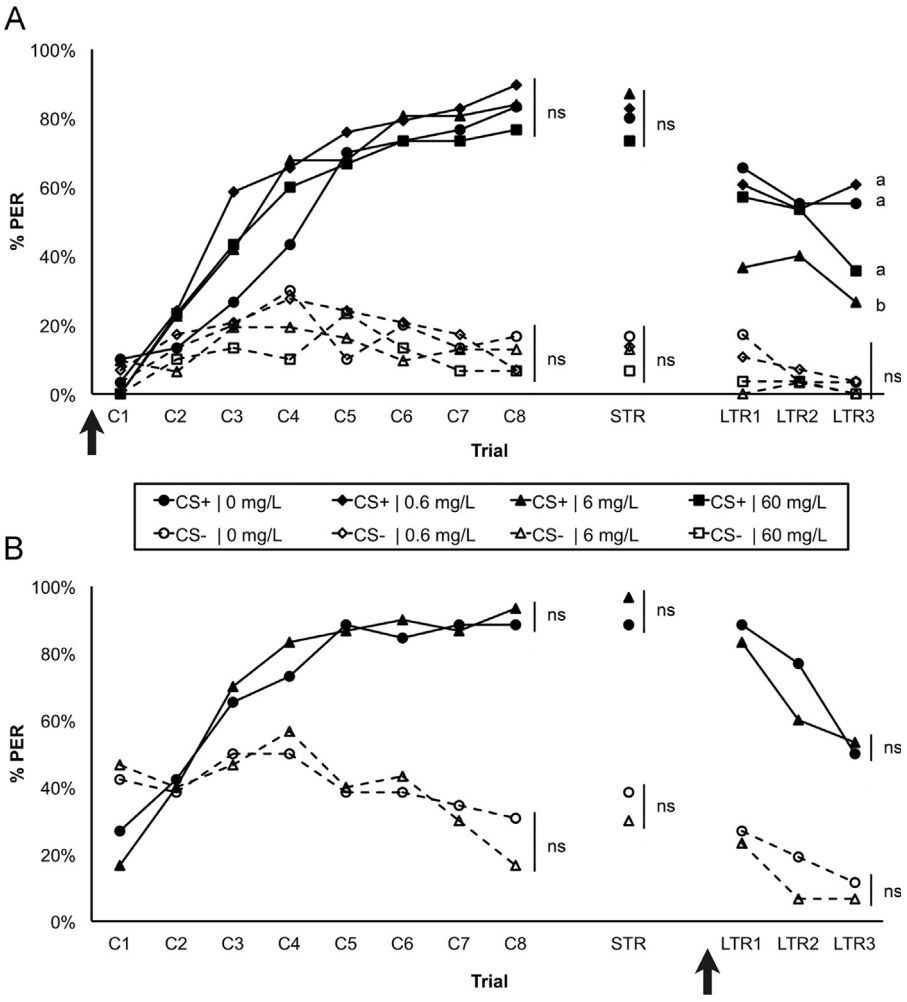

Fig. 2. The percentage of methylseleno-L-cysteine treated bees exhibiting PER (% PER) to each trial during acquisition trials (C1–C8), test trials immediately following conditioning (STR), and test trials approximately 24 h following conditioning (LTR1–LTR3). Bees were either treated with methylseleno-L-cysteine 3 h prior to conditioning (A; for C1–C8 & STR: $n=28$–31, for LTR1–LTR3: $n=28$–30) or 3 h prior to the long-term test trials (B; for C1–C8 & STR: $n=25$–30, for LTR1–LTR3: $n=26$–30). CS+ indicates the sucrose-reinforced odor, while CS− represents the unreinforced odor. The arrows indicate the timing of selenium treatment.

Table 4
Logistic Generalized Estimating Equations analysis of the bees' responses during conditioning, short-term recall, and long-term recall of bees treated with methylseleno-L-cysteine 3 h prior to beginning conditioning. The bolded *p*-values are those significantly correlated with the probability of bees exhibiting PER to the conditioning or test trials.

		Conditioning		Short-term Recall		Long-term Recall	
Predictor	**Contrast**	β^1		β^1		β^1	
		Wald χ^2	***p-Value***	***Wald χ^2***	***p-Value***	***Wald χ^2***	***p-Value***
ODOR	CS− vs. CS+	2.255		3.444		3.148	
		175.512	***0.000***	*97.999*	***0.000***	*86.878*	***0.000***
DOSE	0 mg/L vs. 0.6 mg/L	0.384		−0.004		−1.179	
		1.155	*0.282*	*0.000*	*0.994*	*0.651*	*0.420*
DOSE	0 mg/L vs. 6 mg/L	0.113		0.129		−4.469	
		0.105	*0.746*	*0.061*	*0.805*	*7.357*	***0.007***
DOSE	0 mg/L vs. 60 mg/L	−0.095		−0.633		−2.926	
		0.075	*0.784*	*1.394*	*0.238*	*3.453*	*0.063*
TRIAL		1.392		–––		−2.046	
		92.862	***0.000***			*4.261*	***0.039***
TRIAL^2		−0.110		–––		0.409	
		66.374	***0.000***			*3.123*	0.077
DOSE × TRIAL	0 mg/L vs. 0.6 mg/L	–––		–––		0.961	
						0.433	*0.511*
DOSE × TRIAL	0 mg/L vs. 6 mg/L	–––		–––		3.742	
						4.678	***0.031***
DOSE × TRIAL	0 mg/L vs. 60 mg/L	–––		–––		2.920	
						3.148	*0.076*
DOSE × TRIAL^2	0 mg/L vs. 0.6 mg/L	–––		–––		−0.168	
						0.242	*0.623*
DOSE × TRIAL^2	0 mg/L vs. 6 mg/L	–––		–––		−0.889	
						4.348	***0.037***
DOSE × TRIAL^2	0 mg/L vs. 60 mg/L	–––		–––		−0.741	
						3.382	*0.066*

[1] The parameter estimate indicating the relationship of the predictor to the percentage of bees responding during conditioning and recall.

Table 5
Logistic Generalized Estimating Equations analysis of the responses during conditioning, short-term recall, and long-term recall of bees treated with methylseleno-L-cysteine 3 h prior to beginning the long-term recall test. The bolded *p*-values are those significantly correlated with the probability of bees exhibiting PER to the conditioning or test trials.

		Conditioning		Short-term Recall		Long-term Recall	
Predictor	**Contrast**	β^1 ***Wald χ^2***	***p-Value***	β^1 ***Wald χ^2***	***p-Value***	β^1 ***Wald χ^2***	***p-Value***
ODOR	CS− vs. CS+	1.336		3.231		2.706	
		73.506	***0.000***	*35.872*	***0.000***	*76.957*	***0.000***
DOSE	0 mg/L vs. 6 mg/L	0.071		−0.009		−0.411	
		0.017	*0.897*	*0.000*	*0.987*	*1.006*	*0.316*
TRIAL		0.861		–––		−1.408	
		34.189	***0.000***			*2.516*	*0.113*
TRIAL^2		−0.078		–––		0.159	
		26.490	***0.000***			*0.536*	*0.464*

[1] The parameter estimate indicating the relationship of the predictor to the percentage of bees responding during conditioning and recall.

significant difference between the two groups before treatment with methylseleno-L-cysteine, as was expected.

We then tested the bees' memory of the task with recall tests. During the short-term recall test there was no significant difference between the performances of two groups of bees (Fig. 2B, Table 5). Both groups discriminated well between the CS+ and the CS−. Following exposure to methylseleno-L-cysteine, the bees still discriminated well between the CS+ and the CS−. There was no difference between the methylseleno-L-cysteine treated bees performance on the long-term recall trials.

Following the short- and long-term recall tests we determined the bees' response levels to antennal stimulation with sucrose. There was no difference in the percentage responding to the sucrose stimulation between bees treated with methylseleno-L-cysteine and controls following the short- and long-term recall tests (Table 2; Pearson's Chi-square, short-term recall: $\chi^2=1.175$, d$f=1$, $p=0.278$, long-term recall: $\chi^2=0.463$, d$f=1$, $p=0.496$).

4. Discussion

Acute treatment with sublethal dosages of selenium affects a honey bee's performance during acquisition and/or recall of learned olfactory information. This reduction could occur because of interference with the ability to distinguish between the rewarded and unreinforced odors, or because of an overall reduction in the response to olfactory stimuli, especially seen in the response to the rewarded odor. The reduction may be due to impairment in sensory detection of the olfactory stimulus or through interference in one or more of the neural processes involved in learning,

memory consolidation, and memory recall. It did not seem to be due to interference with motor processes involved in PER or to reduction in the motivation to feed. Bees showed normal behavioral responses to sucrose in spite of showing reductions in responses to conditioned odors.

The effect of selenium treatment depended on the phase of conditioning and testing as well as on the form of selenium. During the initial acquisition phase, bees that ingested a single dose of sodium selenate before the beginning of the conditioning trials exhibited a decrease in their responsiveness to the CS+, and sometime to the CS−, during olfactory discrimination conditioning. Bees ingesting a dose of methylseleno-L-cysteine, a reportedly less toxic form of selenium (Quinn et al., 2011), did not show this impairment during conditioning. Interestingly, there was no significant difference between selenium treated bees and controls during the short-term recall test for either form of selenium, which could indicate at least some recovery 30 min after acquisition. In spite of this apparent early recovery, the largest effect of selenium treatment – for either form – was during the long-term recall test. The bees treated with moderately high doses (6 mg/L) of either form of selenium prior to conditioning exhibited decreased performance during the long-term recall test.

The absence of a treatment effect during the short-term recall test, and the emergence of an effect during the long-term test, may be because more time is required for selenium to be absorbed and exert its toxic effect than the 3 h between selenium exposure and conditioning and the short-term recall test. By the time of the long-term recall test, sufficient damage from the toxic levels of selenium may have occurred to alter the bees' behavior. Alternatively, the mechanisms targeted by selenium toxicity may be those involved in long-term memory consolidation or recall, leaving short-term memory relatively unimpaired.

Unexpectedly, for both sodium selenate and methylseleno-L-cysteine, the bees fed the highest dose (60 mg/L) did not show significant deficits in their performance on either conditioning trials or the short- and long-term recall trials. This unusual type of u-shaped dose-response curve has been identified in previous neurotoxicology studies, though the underlying mechanisms remain elusive (Bleecker et al., 1997; Davis and Svendsgaard, 1990; Davis et al., 1990). Consequently, any interpretation of the u-shaped dose response curve must be given with caution. One possible explanation, however, is that honey bees may have some type of physiological compensatory mechanism that either combats or masks the effects of selenium toxicity at this high yet sublethal dosage.

Following the short- and long-term recall trials, we performed a sucrose responsiveness assay to determine if the performance of bees exposed to selenium could be attributed to reduced motivation or ability to respond with PER to the odor stimulus. We did not observe a decreased responsiveness to sucrose stimulation in selenium treated animals compared to controls; however, we used a relatively high sucrose content stimulus, which typically elicits very strong PER. The conditioned response of PER to test trial odor stimulation is a sensitive measure of motivation and thus could be detecting a moderate decline in motivation or the ability to respond to sucrose stimulation that could not be resolved with the sucrose responsiveness test following the test trials.

Our data clearly show an effect of sublethal dosages of selenium on a behavior that is important for colony performance. Exposure to sublethal selenium can have a significant effect on honey bee learning and memory within 24 h. The small amount of selenium fed to the bees in this study is less than what bees could encounter in contaminated areas. The concentrations used are well within the ranges of selenium observed in the aerial tissues in several plant species grown in selenium contaminated soil in both greenhouse conditions and contaminated locations (Hladun et al., 2013, 2012; Quinn et al., 2011). And the volume fed is much less than the crop load a honey bee could carry.

Our results are consistent with two different mechanisms potentially mediating the effects of toxic selenium exposure on honey bee behavior. In other species, the cellular level mode of action for selenium toxicity depends on the specific form of the selenium compound. In the case of inorganic selenium cellular damage is likely caused by oxidative damage, as has been shown in cultured cell lines and in mammals (Letavayova et al., 2006). With organic forms of selenium, the molecule may be metabolized into selenocysteine that could then be misincorporated into proteins, causing misfolding and impaired cellular function (Hladun et al., 2013). It is likely that selenium toxicity in honey bees is mediated in similar ways. Therefore, the specific mechanisms of the selenium-induced behavioral impairments we observed likely depend on the form of selenium to which the honey bees were exposed.

Selenium toxicity is likely acting in a non-selective manner. So, the specific impairments caused by excess selenium would be influenced by which tissues and organs are exposed to selenium containing compounds. Also, selenium would have a greater impact on organs that are more susceptible to and are less able to repair selenium-induced damage. It may be that the nervous system is simply one of the first organ systems to suffer irreparable damage and thus exhibit impaired functionality. Alternatively, non-neural peripheral toxic effects may also be sources of the selenium induced behavioral impairments through induction of malaise, or compromising function of organs playing a supportive role in brain function and health. Further studies that examine which organs and tissues in the bee are damaged following exposure to selenium and the correlation of this damage with the organ/tissue selenium content are needed to determine the exact mechanism mediating selenium-induced behavioral impairments in our experiments.

Selenium induced learning and memory impairments could impact honey bees' ability to function as pollinators and maintain healthy colonies. While foraging, honey bees must be able to quickly learn the locations and odor profiles of flower patches, from which they gather the nectar and pollen required for colony survival. Disruption or impairment of learning and/or memory could significantly impair the foragers' efficiency in gathering these resources and their ability to pollinate the many crops depending on them for good productivity.

As our awareness of these environmental contaminants increases, it is becoming increasingly apparent that we must further our understanding of the harm caused by the plethora of toxins, diseases, pests, etc. that are challenging honey bee populations worldwide. Recent studies have detected the presence of multiple pesticides, heavy metals, and metalloids in honey bee colonies throughout the U.S. and Canada, some of which are already known to have negative impacts on honey bee behavior at the detected concentrations (Mullin et al., 2010; Pettis et al., 2013; Søvik et al., 2015). However, for most of the pesticides and other toxic chemicals present in the honey bees' environment, there is still little known about how sublethal levels of these chemicals affect the behavior of honey bees and other pollinators.

Identifying changes in behavior caused by a toxin will allow us to identify the sublethal concentrations at which honey bees first become impaired so we can work toward sufficiently cleaning highly contaminated areas and setting safe limits for toxin and pesticide presence in the environment. There may be interactions between these toxins and other challenges to honey bee health that could augment the influence an individual toxin or disease has on behavior and colony survival, so furthering our understanding of these potential synergistic relationships is of great import as well.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2015.12.034.

References

Bleecker, M.L., Lindgren, K.N., Tiburzi, M.J., Ford, D.P., 1997. Curvilinear relationship between blood lead level and reaction time. Differential association with blood lead fractions derived from exogenous and endogenous sources. J. Occup. Environ. Med. 39, 426–431. http://dx.doi.org/10.1097/00043764-199705000-00008.

Brown Jr., G.E., Foster, A.L., Ostergren, J.D., 1999. Mineral surfaces and bioavailability of heavy metals : A molecular-scale perspective. Proc. Natl. Acad. Sci. USA 96, 3388–3395.

Davis, J.M., Otto, D., AWeil, D.E., Grant, L.D., 1990. The comparative developmental neurotoxicity of lead in humans and animals. Neurotoxicol. Teratol. 12, 215–229. http://dx.doi.org/10.1016/0892-0362(90)90093-R.

Davis, J.M., Svendsgaard, D.J., 1990. U-shaped dose-response curves: their occurrence and implications for risk assessment. J. Toxicol. Environ. Health 30, 71–83. http://dx.doi.org/10.1080/15287399009531412.

El Mehdawi, A.F., Pilon-Smits, E.A.H., 2012. Ecological aspects of plant selenium hyperaccumulation. Plant Biol. 14, 1–10. http://dx.doi.org/10.1111/j.1438-8677.2011.00535.x.

Hladun, K.R., Kaftanoglu, O., Parker, D.R., Tran, K.D., Trumble, J.T., 2013. Effects of selenium on development, survival, and accumulation in the honeybee (*Apis mellifera* L.). Environ. Toxicol. Chem. 32, 2584–2592. http://dx.doi.org/10.1002/etc.2357.

Hladun, K.R., Smith, B.H., Mustard, J.A., Morton, R.R., Trumble, J.T., 2012. Selenium toxicity to honey bee (*Apis mellifera* L.) pollinators: effects on behaviors and survival. Plos. One 7, e34137. http://dx.doi.org/10.1371/journal.pone.0034137.

Lakin, H.W., 1972. Selenium accumulation in soils and its absorption by plants and animals. Geol. Soc. Am. Bull. 83, 181–190.

Letavayova, L., Vlckova, V., Brozmanova, J., 2006. Selenium: from cancer prevention to DNA damage. Toxicology 227, 1–14. http://dx.doi.org/10.1016/j.tox.2006.07.017.

Mehdi, Y., Hornick, J., Istasse, L., Dufrasne, I., 2013. Selenium in the environment, metabolism and involvement in body functions. Molecules 18, 3292–3311. http://dx.doi.org/10.3390/molecules18033292.

Mullin, C.A., Frazier, M., Frazier, J.L., Ashcraft, S., Simonds, R., VanEngelsdorp, D., Pettis, J.S., 2010. High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. PLoS One 5, e9754. http://dx.doi.org/10.1371/journal.pone.0009754.

Pettis, J.S., Lichtenberg, E.M., Andree, M., Stitzinger, J., Rose, R., VanEngelsdorp, D., 2013. Crop pollination exposes honey bees to pesticides which alters their susceptibility to the gut pathogen *Nosema ceranae*. PLoS One 8, e70182. http://dx.doi.org/10.1371/journal.pone.0070182.

Quinn, C.F., Prins, C.N., Freeman, J.L., Gross, A.M., Hantzis, L.J., Ray, J.B., Yang, S., Covey, P.A., Banuelos, G.S., Pickering, I.J., Fakra, S.C., Markus, M.A., Arathi, H.S., Pilon-Smits, E.A.H., 2011. Selenium accumulation in flowers and its effects on pollination. New Phytol. 192, 727–737. http://dx.doi.org/10.1111/j.14698137.2011.03832.x.

Smith, B.H., Abramson, C.I., Tobin, T.R., 1991. Conditional withholding of proboscis extension in honeybees (*Apis mellifera*) during discriminative punishment. J. Comp. Psychol. 105, 345–356. http://dx.doi.org/10.1037/0735-7036.105.4.345.

Smith, B.H., Burden, C.M., 2014. A proboscis extension response protocol for investigating behavioral plasticity in insects: application to basic, biomedical, and agricultural research. J. Vis. Exp. 91, e51057. http://dx.doi.org/10.3791/51057.

Søvik, E., Perry, C.J., Lamora, A., Barron, A.B., Ben-shahar, Y., 2015. Negative impact of manganese on honeybee foraging. Biol. Lett. 11, 20140989.

Thorn, R.S., Smith, B.H., 1997. The olfactory memory of the honeybee *Apis mellifera* III. Bilateral sensory input is necessary for induction and expression of olfactory blocking. J. Exp. Biol. 200, 2045–2055.

Wright, G.A., Lutmerding, A., Dudareva, N., Smith, B.H., 2005. Intensity and the ratios of compounds in the scent of snapdragon flowers affect scent discrimination by honeybees (*Apis mellifera*). J. Comp. Physiol. A 191, 105–114. http://dx.doi.org/10.1007/s00359-004-0576-6.

Wright, G.A., Thomson, M.G.A., Smith, B.H., 2009. Odour concentration affects odour identity in honeybees. Proc. R. Soc. B 272, 2417–2422. http://dx.doi.org/10.1098/rspb.2005.3252.