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EFFECTS OF SELENIUM ON DEVELOPMENT, SURVIVAL, AND ACCUMULATION IN THE HONEYBEE (*APIS MELLIFERA* L.)

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Abstract: *Apis mellifera* L. (Hymenoptera: Apidae) is an important agricultural pollinator in the United States and throughout the world. In areas of selenium (Se) contamination, honeybees may be at risk because of the biotransfer of Se from plant products such as nectar and pollen. Several forms of Se can occur in accumulating plants. In the present study, the toxicity of 4 compounds (selenate, selenite, methylselenocysteine, and selenocystine) to honeybee adult foragers and larvae was assessed using dose–response bioassays. Inorganic forms were more toxic than organic forms for both larvae (lethal concentration [LC50] selenate = 0.72 mg L^{-1}, LC50 selenite = 1.0 mg L^{-1}, LC50 methylselenocysteine = 4.7 mg L^{-1}, LC50 selenocystine = 4.4 mg L^{-1}) and foragers (LC50 selenate = 58 mg L^{-1}, LC50 selenite = 58 mg L^{-1}, LC50 methylselenocysteine = 161 mg L^{-1}, LC50 selenocystine = 148 mg L^{-1}). Inorganic forms of Se caused rapid mortality, and organic forms had sublethal effects on development. Larvae accumulated substantial amounts of Se only at the highest doses, whereas foragers accumulated large quantities at all doses. The present study documented very low larval LC50 values for Se; even modest transfer to brood will likely cause increased development times and mortality. The toxicities of the various forms of Se to honeybee larvae and foragers are discussed in comparison with other insect herbivores and detritivores. *Environ Toxicol Chem* 2013;32:2584–2592.

Keywords: Methylselenocysteine Pollinator Selenate Selenite Selenocystine

INTRODUCTION

Selenium contamination is a global problem originating from a multitude of sources, including mine tailings and production of glass, pigments, inks, and lubricants, as well as the concentration of Se in drainage water through agricultural irrigation or rainfall on naturally seleniferous soils [1]. In the western United States, more than 607 000 hectares are contaminated [2]. Imported irrigation water containing low concentrations of salts is applied to farmland and leaches naturally occurring soil minerals such as Se. The well-established toxicity of Se to wildlife and humans has caused this element to be regulated by the Toxic Substances Control Act [3] and the Clean Water Act [4]. Once an endpoint for the drainage of Se-contaminated waters, the Kesterson National Wildlife Refuge serves as an example of the toxicological effects of Se on wildlife, with a 64% rate of deformity and death of embryos and hatchlings of wild birds. Contaminated water entering the Kesterson Reservoir averaged 0.3 mg Se L^{-1}, and soil concentrations reached as high as 8 mg Se kg^{-1} [5]. Topsoils and efflorescences in the Central Valley of California, USA, can reach as high as 50 mg Se kg^{-1} [2]. Similar situations exist farther south in the Tulare Lake Bed area, the Salton Sea area, and 9 other areas in the western United States [6]. Throughout the central and eastern United States, power plants produce coal-fly ash that can create Se-contaminated environments [7].

Selenium has several different oxidation states, including selenate (Se^{+6}), selenite (Se^{+4}), elemental Se (Se^{0}), and selenides or organic forms of Se (Se^{+2}). In most cases, sodium selenate is transported via agricultural irrigation water and then transformed within plants to the organic forms, methylselenocysteine and selenocysteine [8]. The toxic effects of these selenoamino acids are likely attributed to replacement of sulfur with Se in methionine and cysteine, resulting in protein misfolding and consequently nonfunctional proteins and enzymes [9,10]. Selenium bioaccumulation has been documented in *Chironomus decorus* Johannsen (Diptera: Chironomidae) [7,11], *Trichocorixa reticulata* (Guerin-Meneville; Hemiptera: Corixidae) [12], *Musca domestica* L. (Diptera: Muscidae) [13], *Spodoptera exigua* (Hübner; Lepidoptera: Noctuidae) [14], and *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) [15]. In all cases, Se accumulation occurred through oral exposure. No studies have compared Se toxicity in the adults or larvae of Apidae.

As a result of the extensive terrestrial contamination, the use of plants to accumulate and disperse Se through phytoremediation has developed into an important strategy for land reclamation [1,16]. Such large-scale Se accumulation by phytoremediating plant species has the potential to alter local ecosystems and may adversely affect plant mutualists such as pollinators. In addition, 2 common weed species (radish, *Raphanus sativus* L., and mustard, *Brassica juncea* Czern L.) are capable of accumulating very high levels of Se in the pollen and nectar when grown in the greenhouse. Nectar contained up to 110 mg Se L^{-1}, and pollen contained 710 mg Se kg^{-1} and 1700 mg Se kg^{-1} in *B. juncea* and *R. sativus*, respectively [17,18]. In a semi-field study, honeybees (*Apis mellifera* L., Hymenoptera: Apidae) foraged on *R. sativus* that contained whole flower concentrations up to 219 mg Se kg^{-1} dry weight (dw) [18]. In a naturally seleniferous landscape, both honeybee and bumblebee (*Bombus* sp.) pollinators have been observed to forage on accumulating plants with concentrations as high as

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3200 mg Se kg^{-1} in the flowers [19]. Based on the concentrations listed, honeybees have the potential to bring food resources back to the hive that are contaminated with Se at levels shown to be toxic to other insect species. Selenium in honey may be biotransferred from nurse bees to the developing brood as a contaminated food source. Honey from different regions of Turkey contained 0.04 to 0.11 mg Se kg^{-1} [20], and honey collected from hives located in seleniferous areas of Colorado, USA, contained up to 0.73 mg Se kg^{-1} [19]. Certain species of larval herbivores do not avoid Se in plant tissues [14] and may not detect certain forms of Se [21]. Therefore, these insects can ingest toxic levels of the element. Honeybee adult foragers are not deterred from feeding on sucrose solutions containing various concentrations and forms of Se [22], and if larvae display a similar lack of feeding inhibition, they may consume detrimental quantities of Se in their diet as well.

Several forms of Se can occur in the flowers of accumulating plants, particularly selenate, selenite, methylselenocysteine, selenocysteine and selenocystine. Methylselenocysteine and selenate were the predominant forms found in *R. sativus* [23] and *B. juncea* [19], but selenocystine and selenite were also present in smaller quantities [19]. Therefore, the Se compounds used in the present study included 2 inorganic forms, selenate and selenite, as well as 2 organic forms, methylselenocysteine and selenocystine. These forms were chosen for comparison with toxicity assays using *S. exigua* [14,21,24] and *Megaselia scalaris* (Loew; Diptera: Phoridae) [25]. Doses contained ecologically relevant concentrations for honeybee larvae (up to 10 mg Se L^{-1}) and adults (up to 480 mg Se L^{-1}) and were based on the concentrations found in the nectar, pollen, or honey from greenhouse- or field-grown plants as well as honeybee hives listed in the studies previously mentioned. For the present study, we reared honeybees in vitro using a chronic feeding assay that provisioned the larvae once [26] with Se-laced artificial diet. Although the lethal concentration that kills 50% of the *A. mellifera* population (LC50) can be used to compare the susceptibility among species, bioassay parameters that consider more than just survivorship can reveal the more subtle, sublethal effects of a toxin. Exposing the species at different life stages may reveal different susceptibilities [27]. In addition, chronic exposure represents a more realistic scenario experienced by bees when foraging in a contaminated area. In the present study, we exposed honeybees to Se during all larval stages and collected both mortality and development data daily. The primary objective of the present study, therefore, was to determine whether 4 forms of Se found in floral tissues of accumulating plants can have a detrimental effect on the development and survival of both adult foragers and larvae in a common pollinator, the honeybee, *A. mellifera*.

MATERIALS AND METHODS

Materials

Sodium selenate (henceforth, selenate; Na_2SeO_4, 98% purity), sodium selenite (henceforth, selenite; Na_2SeO_3, 99% purity) and seleno-L-cystine (henceforth, selenocystine; 97% purity) were purchased from Sigma-Aldrich. Selenium-methyl-seleno-L-cysteine (henceforth, methylselenocysteine; 98% purity) was purchased from Acros Organics.

Larval bioassays

Tests were performed in 2012 and 2013 at the University of California–Riverside (Riverside, CA, USA) using honeybee (*A. mellifera ligustica*) foragers collected from 2 hives maintained at Agricultural Operations at the University of California–Riverside. The queens were not changed during the course of these experiments to minimize genetic variation. Using methods based on Peng et al. [28] and Aupinel et al. [29], the queen was confined to a frame containing empty cells using an excluder cage for 24 h. The cage allowed workers to move freely from the confined frame and the surrounding colony, while preventing the queen from leaving the cage. This method ensured eggs of a similar age. The queen was then removed and the frame replaced in the cage to prevent any further oviposition. The frame was removed 4 d later, and the resulting 1-d-old larvae were removed from wax cells and placed onto artificial diet using a grafting tool.

Several recent studies have standardized the methods for rearing *A. mellifera* larvae on artificial diet for the purposes of assessing contaminant toxicity. Laboratory in vitro feeding assays are preferred over in vivo rearing within the colony to accurately administer doses in a known quantity of food [30]. Mortality can be reduced in the bioassay through minimal handling [31] as well as a single, mass provisioning of food to sustain the insect throughout larval development [26]. Adding other components to the royal jelly, such as sugars and yeast extract, can also provide sustenance similar to nectar for brood growth and energy [26,29]. The improvements in technique for rearing *A. mellifera* larvae in vitro have made laboratory toxicity tests more reliable. Adding Se to the sugar solution component of the diet can mimic contaminated nectar.

Artificial diet was prepared as described in Kaftanoglu et al. [26]. The diet consisted of 53% (w/w) commercial frozen royal jelly, 6% glucose, 6% fructose, 1% yeast extract [32], and 34% distilled water. The Se compounds were dissolved into the sugar solution portion to yield final target concentrations in the diet. All grafting tools, petri plates, cell cups, and well plates were ultraviolet sterilized before use to minimize contamination (Air Clean 600 PCR Workstation; ISC Bioexpress). Larvae were provisioned once with 200 mg artificial diet placed inside of queen cell cups (Glory Bee Foods, Inc., Eugene, OR). After grafting, petri dishes were kept in an incubator at 34.1 ± 0.1 °C and 92.7 ± 0.4% relative humidity in constant darkness. The confounding effect of damage caused by handling was eliminated by removing individuals that had died within 1 d after grafting. At the prepupal stage on d 10, larvae were weighed and moved to 24-well plates (Costar 3526 cell culture plates; Corning) to allow more space for pupation.

Larval diet spiked with Se was prepared in 6 concentrations as selenate or selenite: 0 mg Se L^{-1}, 0.2 mg L^{-1}, 0.4 mg L^{-1}, 0.6 mg L^{-1}, 1 mg L^{-1}, and 2 mg L^{-1}. For methylselenocysteine, the concentrations tested were 0 mg Se L^{-1}, 4 mg L^{-1}, 6 mg L^{-1}, 7 mg L^{-1}, 9 mg L^{-1}, and 10 mg L^{-1}. For selenocystine, larvae were fed diets containing 0 mg Se L^{-1}, 2 mg L^{-1}, 4 mg L^{-1}, 6 mg L^{-1}, 8 mg L^{-1}, and 10 mg L^{-1}. These concentrations were chosen based on preliminary range-finding tests. Mortality was scored daily. The LC50 was calculated for each Se form to determine which forms were most toxic and to compare the LC50s with other insects from different feeding guilds. At least 3 replicates per treatment level (4 forms $\times$ 6 concentrations containing up to 27 larvae each) were grafted onto contaminated diet on day 4 (when larvae were approximately 1 d old) [31]. We calculated the LC50 values after chronically feeding the larvae for 8 d. Day 8 was chosen for the relative toxicity values for all 4 Se forms to mimic chronic exposure to Se that a terrestrial insect may encounter [33].

Prepupae were weighed using a microbalance (weighing to 0.00001 g; model 1712 MP8; Sartorius Corp.) on d 10. The number of individuals that survived to the prepupal and pupal stages was used to calculate the percentage of prepupation and pupation.

To determine the potential changes in growth rates, we calculated a relative growth index (RGI), a measure of the distribution of treated individuals in each instar, which may vary from individuals fed the control diet. Growth inhibition can cause more individuals to remain in earlier instars and may be concentration dependent [34]. Selenium delays development and growth in both *S. exigua* [21] and *Culex quinquefasciatus* Say (Diptera: Culicidae) [33]. Honeybee larvae were scored as being in 1 of 3 developmental stages, based on descriptions by Snodgrass and Erickson [35] and Winston [36]: larva (instars 1 through 4), prepupa (the last larval instar, characterized by spinning of the cocoon), or pupa. The numbers of live and dead individuals in these 3 stages were scored on a daily basis for up to 10 d. The RGI was calculated for days 4 through 10 using equations described by Zhang et al. [34].

Forager bioassays

Adult nectar foragers were collected at the entrances of 2 hives, harnessed with duct tape in a straw tube holder so that only the head, antennae, and proboscis were free, and fed to satiation 24 h before dosing [22]. Foragers from both hives were mixed and haphazardly distributed in each treatment group. The queens were not changed during the study. Oral toxicity tests were based on US Environmental Protection Agency (USEPA) guidelines for the acute oral toxicity testing of honeybees [37]. Bees were dosed with 19.2 μL of the Se treatment. The treatment concentrations used for the inorganic forms of Se were 0 mg Se (as selenate or selenite) L^{-1}, 30 mg L^{-1}, 60 mg L^{-1}, 120 mg L^{-1}, 240 mg L^{-1}, and 480 mg L^{-1}. The Se compounds were dissolved in 50% sucrose solution. The treatment concentrations used for the organic forms of Se were 0 mg Se (as methylselenocysteine or selenocystine) L^{-1}, 104 mg L^{-1}, 125 mg L^{-1}, 150 mg L^{-1}, 200 mg L^{-1}, and 250 mg L^{-1}. After dosing at 0 h, bees were scored for mortality 24 h, 48 h, and 72 h postdosing. Each day, bees were then fed to satiation with 50% sucrose solution using a micrometer glass syringe (Gilmont Instruments). The total volume of sucrose consumed was recorded.

Selenium analyses

Prepupal, pupal, and adult forager tissues were frozen in a −60 °C freezer (Fisher Scientific) and then freeze-dried (Labconco Corp) at −40 °C and −25 psi for at least 3 d. All freeze-dried insect tissues were stored in a −60 °C freezer until digestion and future Se analysis. Up to 20 individual insects were pooled within a replicate to create a sufficient tissue weight for analysis. Insect tissues were weighed using a microbalance before microwave digestion. Insect tissues were then microwave digested in 110-mL Teflon-lined vessels containing 5 mL concentrated HNO_3 [38]. The vessels were heated for 20 min using a 570-W microwave oven (CEM Corp). Insect tissue digestates were then diluted in a 6-M HCl matrix, heated in a 90 °C water bath for 20 min, and analyzed using hydride vapor-generated atomic absorption spectroscopy. The National Institute of Standards and Technology (NIST) standard reference material 1566B (oyster tissue, a common invertebrate standard) was used to verify recovery from a similar biological matrix. All duplicate sample concentrations were within 10% of each other, and Se spike recovery and NIST Se recovery were more than 90%.

Statistical analysis

Each Se form was analyzed as a separate independent variable. The responses analyzed were prepupal weight, prepupation percentage, and pupation percentage, using general linear models (PROC GLM, SAS 9.2; SAS Institute) with type III sum of squares. Mean separations were conducted between groups ($\alpha = 0.05$) using post hoc Tukey's honestly significant difference (HSD) test. Assumptions of normality were examined using the Shapiro-Wilks test, and all response data listed above met assumptions of normality and homogeneity of variance.

Relative growth indices were analyzed using analysis of variance (ANOVA; PROC GLM) with repeated measures. The independent variable was Se treatment concentration, RGI was the dependent variable, and day (over the 10 d of development) was the repeated variable. Mean separations were conducted between groups ($\alpha = 0.05$) using post hoc Tukey's HSD test. Each Se treatment concentration was repeated with at least 3 replicates, each containing 27 larvae.

As recommended in the USEPA ecological effects test guidelines for honeybee toxicity assays [37], mortality was 20% or less in all control groups. Abbott's formula was used to correct for control mortality [39]. For LC50 larval bioassays, at least 3 replicates were used for each treatment. The LC50s were calculated using probit analysis (PROC PROBIT, SAS 9.2; SAS Institute). The LC50s were modeled with normal distributions for selenate, selenite, and methylselenocysteine. Selenocystine was modeled with a Gompertz distribution.

At least 3 replicates were used for each LC50 forager bioassay treatment. Each replicate consisted of 19 foragers. The LC50s were calculated using probit analysis (PROC PROBIT, SAS 9.2; SAS Institute) and were modeled with normal distributions for selenate, selenite, methylselenocysteine, and selenocystine. The volume of sucrose consumed after dosing was analyzed with ANOVA (PROC GLM, SAS 9.2; SAS Institute), with type III sum of squares; the independent variables were Se treatment dose and hour, and sucrose consumption was the dependent variable. Each Se form was analyzed separately. Sucrose consumption was square root transformed for selenate to achieve normal distribution. Selenium accumulation data for selenite-fed foragers were log transformed. Post hoc comparisons were made using Tukey's HSD tests.

RESULTS

Prepupal weight, percentage of prepupation, and pupation

None of the 4 forms of Se had a significant effect on prepupal weight (ANOVA, $F < 0.47$, $p > 0.78$ for all). However, Se significantly decreased the percentage of larvae that reached the prepupal stage. Selenate (ANOVA, $F_{5,24} = 19$, $p < 0.001$) and selenite (ANOVA, $F_{5,8} = 8.0$, $p < 0.01$) significantly reduced the prepupation percentage (Figure 1A). Selenate (ANOVA, $F_{4,18} = 3.6$, $p < 0.03$) but not selenite (ANOVA, $F_{4,5} = 0.39$, $p = 0.81$) significantly reduced the pupation percentage. None of the larvae pupated in the selenite treatment groups. Pupation significantly dropped from 74% in the controls to 9% in the 1 mg L^{-1} selenate treatment group (Tukey HSD test, $p < 0.05$). Methylselenocysteine (ANOVA, $F_{5,12} = 9.1$, $p < 0.001$) and selenocystine (ANOVA, $F_{5,12} = 13$, $p < 0.001$) reduced the percentage of larvae that reached the prepupal stage by up to 95% and 68%, respectively (Tukey HSD test, $p < 0.05$; Figure 1B).

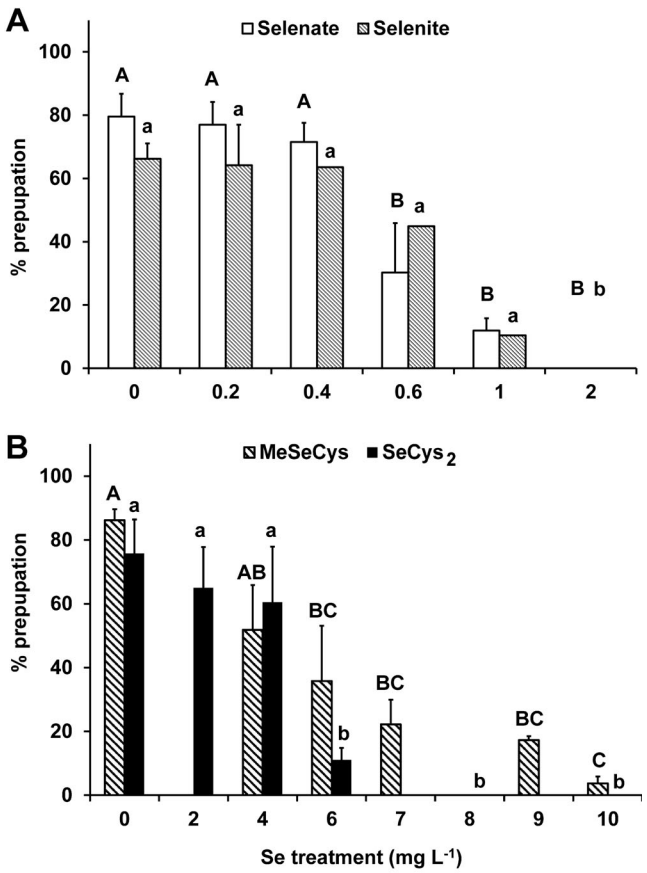

Figure 1. Effects of Se on the percent prepupation of *A. mellifera* larvae fed artificial diet containing **(A)** inorganic forms of Se (selenate ($n = 5$) and selenite ($n = 5$)), and **(B)** organic forms of Se (methylselenocysteine (MeSeCys) ($n = 3$) and selenocystine ($SeCys_2$) ($n = 3$)). Bars (mean ± SE) with the same case and letter are not significantly different within Se species at the $p < 0.05$ level (ANOVA, Tukey's HSD). Means with no error bars denote treatment groups with only one replicate.

For the individuals that survived to prepupal stage, neither methylselenocysteine (ANOVA, $F_{5,11} = 2.4$, $p = 0.10$) nor selenocystine (ANOVA, $F_{3,8} = 2.3$, $p = 0.15$) had a significant effect on pupation.

Relative growth indices

The relative growth indices (RGI) were calculated for *A. mellifera* larvae exposed to selenate, selenite, methylselenocysteine, and selenocystine and are shown for days 4 through 10 (Figure 2). For selenate, day (ANOVA, $F_{8,192} = 6.1$, $p < 0.001$), Se treatment (ANOVA, $F_{5,24} = 14$, $p < 0.001$), and the interaction of day and Se treatment (ANOVA, $F_{40,192} = 6.1$, $p < 0.001$) had an overall significant effect on RGI. There was no significant difference between treatments until day 7. The 2 mg L^{-1} selenate treatment had a significantly lower RGI compared with all other treatments (Tukey HSD test, $p < 0.05$, Figure 2A). By day 9, the 0.6 mg L^{-1}, 1 mg L^{-1}, and 2 mg L^{-1} selenate treatments had significantly lower RGIs compared with the control.

For selenite, day (ANOVA, $F_{8,144} = 9.02$, $p < 0.001$) and Se treatment (ANOVA, $F_{5,18} = 9.51$, $p < 0.001$) had a significant effect on RGI. However, no interaction of day and Se treatment (ANOVA, $F_{40,144} = 0.99$, $p = 0.49$) (Figure 2B) was seen, indicating that there was no significant difference between treatments across all days. The 2 highest selenite treatments (1 mg L^{-1} and 2 mg L^{-1}) significantly reduced relative growth indices overall.

For methylselenocysteine, day (ANOVA, $F_{8,96} = 3.8$, $p < 0.01$), Se treatment (ANOVA, $F_{5,12} = 11$, $p < 0.001$), and the interaction of day and Se treatment (ANOVA, $F_{40,96} = 3.5$, $p < 0.001$) had an overall significant effect on RGI. Larvae fed the control (0 mg L^{-1}) had significantly higher RGIs compared with the 4 mg L^{-1}, 6 mg L^{-1}, 7 mg L^{-1}, 9 mg L^{-1}, and 10 mg L^{-1} methylselenocysteine treatments starting on day 8, and the trend continued until day 10 (Tukey HSD test, $p < .05$, Figure 2C).

For selenocystine, day (ANOVA, Wilks' $\lambda_{8,144} = 9.2$, $p < 0.001$) and Se treatment (ANOVA, $F_{5,18} = 26$, $p < 0.001$) had an overall significant effect on RGI. The interaction of day and Se treatment (ANOVA, $F_{40,144} = 1.2$, $p = 0.31$) was not significant. Larvae fed the control (0 mg L^{-1}) had significantly higher RGI's compared with larvae fed 6 mg L^{-1}, 8 mg L^{-1}, and 10 mg L^{-1} selenocystine (Tukey HSD test, $p < 0.05$, Figure 2D).

Relative toxicity of selenium forms—Larvae

The log–dose probit analysis calculated the concentrations that killed 50% of the population over the entire duration of the larval stages. As indicated by the LC50s, selenate and selenite were the most toxic to honeybee larvae, followed by methylselenocysteine and selenocystine (Table 1). The 95% confidence intervals showed that there was no overlap between inorganic (selenate, selenite) and organic (methylselenocysteine, selenocystine) forms, indicating that inorganic forms were more toxic. All 4 Se forms had lower lethal concentrations for *A. mellifera* larvae compared with most of the other arthropod species (Table 2) with different feeding regimens.

Forager sucrose consumption

Both hour (ANOVA, $F_{2,98} = 19$, $p < 0.001$) and selenate treatment dose (ANOVA, $F_{5,98} = 25$, $p < 0.001$) significantly reduced the volume of sucrose solution the bees consumed after dosing. However, no significant interaction was found between the 2 factors (ANOVA, $F_{8,98} = 0.72$, $p = 0.67$), indicating that selenate did not increase its effect over time. Overall, bees fed at the 24 h time point consumed 4 μL more sucrose than bees fed at the later 2 time points (Tukey, $p < 0.001$). Bees fed 0 mg selenate L^{-1} ingested significantly more sucrose than bees fed any dose of selenate (Figure 3A). Hour (ANOVA, $F_{2,97} = 4.1$, $p < 0.02$) and selenite dose (ANOVA, $F_{5,97} = 28$, $p < 0.001$) also had a significant effect on sucrose consumption. The interaction of the 2 factors was not significant (ANOVA, $F_{10,97} = 0.85$, $p = 1.46$). Control bees (fed 0 mg selenite L^{-1}) consumed more sucrose than selenite-dosed bees (Figure 3A).

Methylselenocysteine treatment dose significantly reduced subsequent sucrose consumption in foragers (ANOVA, $F_{5,83} = 9.4$, $p < 0.001$; Figure 3B). Hour and the interaction of hour and dose were not significant (ANOVA, $F < 2.6$, $p > 0.08$). For selenocystine, hour (ANOVA, $F_{2,86} = 4.4$, $p < 0.02$), dose (ANOVA, $F_{5,86} = 11$, $p < 0.001$), and the interaction of the 2 (ANOVA, $F_{8,86} = 2.4$, $p < 0.03$) were all significant. Bees fed 0 mg L^{-1} or 104 mg L^{-1} selenocystine consumed significantly more sucrose solution than bees fed the higher doses (Tukey, $p < 0.001$; Figure 3B).

Relative toxicity of selenium forms—Foragers

Overall, adult foragers were much more tolerant of Se compared with larvae (Table 1). Similar to larvae, the 95% confidence intervals showed that there was no overlap between inorganic (selenate, selenite) and organic (methylselenocysteine, selenocystine) forms for foragers. Adult foragers had LC50s comparable to *M. scalaris* and *C. decorus* (Table 2).

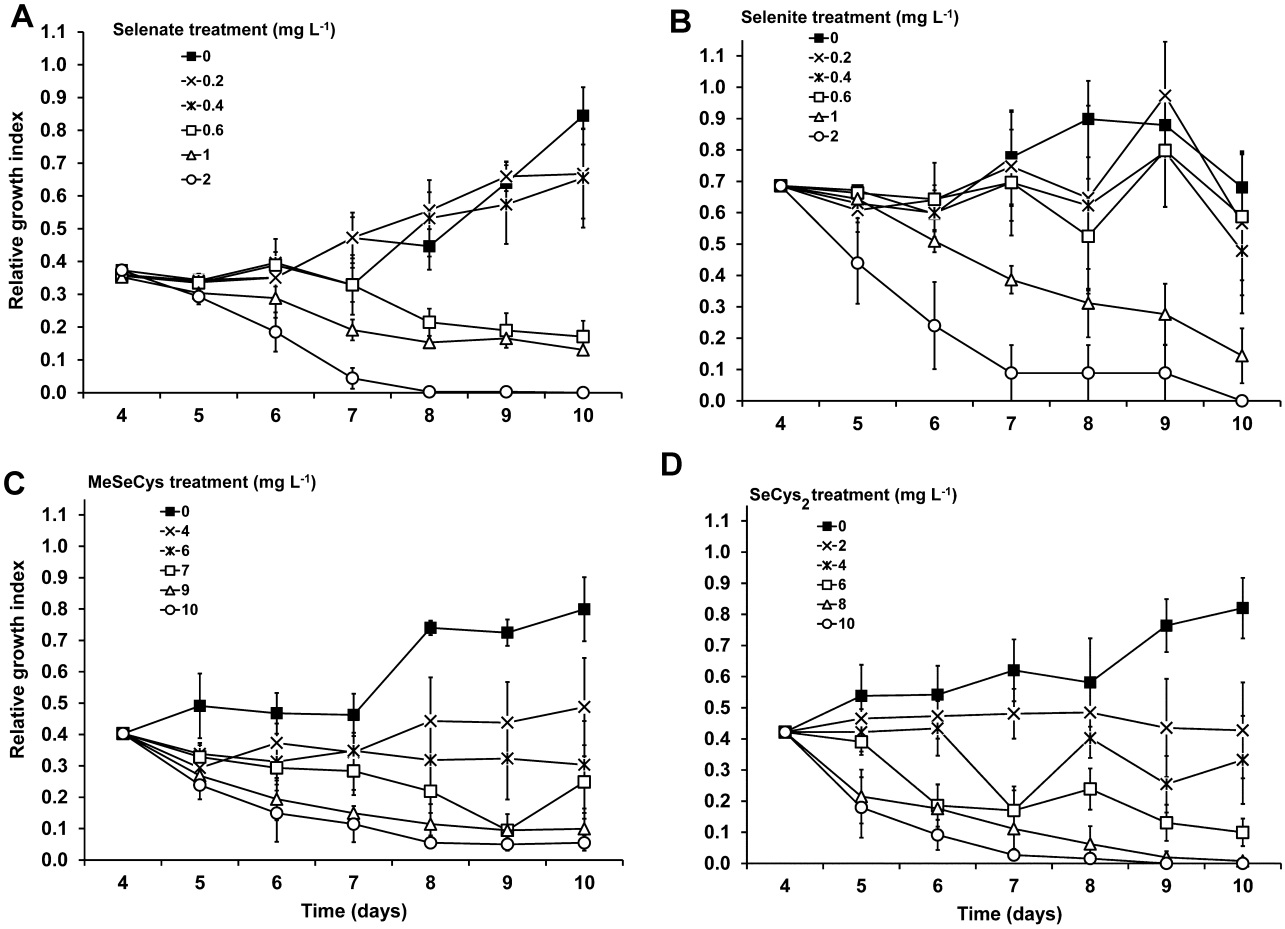

Figure 2. Mean relative growth indices per day of *Apis mellifera* larvae exposed to a range of concentrations for inorganic forms of Se, (**A**) selenate and (**B**) selenite, and organic forms of Se, (**C**) methylselenocysteine (MeSeCys) and (**D**) selenocystine ($SeCys_2$), over a 10-d period. Bars represent standard errors for each treatment on the day of observation (summarized by replicate).

Selenium accumulation in honeybee tissues

Selenate (ANOVA, $F_{3,2} = 6610$, $p < 0.001$), methylselenocysteine (ANOVA, $F_{5,8} = 3.8$, $p < 0.05$), and selenocystine (ANOVA, $F_{3,4} = 0.92, p < 0.05$) doses had a significant effect on Se accumulation in prepupae. Larvae fed selenate accumulated the most Se of all 3 forms analyzed (241 μg Se g^{-1}). Selenium accumulation reached only as high as 19 ± 6 μg Se g^{-1} and 15 μg Se g^{-1} in prepupae when fed methylselenocysteine and selenocystine (Figure 4A), respectively.

Larvae fed selenocystine were the only treatment group that had enough pupal survivors to allow tissue analysis, and no significant effect was seen on Se accumulation (ANOVA, $F_{2,3} = 4.9$, $p = 0.11$). Methylselenocysteine- and selenocystine-fed pupae accumulated up to 11 μg Se g^{-1} ($n = 1$) and 9 μg Se g^{-1} ($n = 1$), respectively. Larvae fed the inorganic forms of Se did not reach the pupal stage.

Table 1. Mean lethal concentrations (LC50) for *Apis mellifera* (Hymenoptera: Apidae) fed four different forms of Se

Selenium form	Number of insects tested	LC50 (mg L^{-1})	95% Confidence limits
Larvae			
Selenate	373	0.72	0.62–0.82
Selenite	268	1.0	0.9–1.3
Methylselenocysteine	486	4.7	4.1–5.3
Seleno-L-cystine	489	4.4	3.2–5.2
Adult foragers			
Selenate	342	58	41–75
Selenite	342	58	37–78
Methylselenocysteine	222	161	141–184
Seleno-L-cystine	252	148	128–168

Foragers fed selenate (ANOVA, $F_{5,12} = 80$, $p < 0.001$) or selenite (ANOVA, $F_{5,23} = 32$, $p < 0.001$) significantly accumulated Se in their tissues at concentrations as high as 230 ± 100 μg Se g^{-1} ($n = 4$; Figure 4B). Selenate accumulation reached its highest concentration in bees fed 480 mg L^{-1}. Selenite accumulation peaked in bees fed the 120 mg Se L^{-1} and did not significantly increase despite the higher doses (Tukey, $p > 0.18$). Selenium dose had a significant effect on accumulation in foragers fed methylselenocysteine (ANOVA, $F_{5,20} = 6.8$, $p < 0.001$) and selenocystine (ANOVA, $F_{5,12} = 3.1$, $p < 0.05$). Bees fed methylselenocysteine accumulated up to 99 ± 18 μg Se g^{-1}, whereas selenocystine accumulated up to 136 ± 34 μg Se g^{-1} (Figure 4C).

DISCUSSION

Apis mellifera is an important agricultural pollinator in the United States and throughout the world. In areas of Se contamination, honeybees may be at risk resulting from the biotransfer of Se from foraged plant products, including nectar and pollen. In particular, honeybee larvae are more susceptible to ingestion of Se-containing food than are adults. The forager's ability to tolerate higher concentrations of Se may act against the colony as a whole. In pesticide toxicity studies, foragers that

Table 2. Comparison of mean lethal concentrations of different Se forms in several insect species[a]

Insect species	Selenium forms	LC50 ($\mu g\ g^{-1}$, $mg\ L^{-1}$)	References
Megaselia scalaris (Diptera: Phoridae)	Selenate	258	Jensen et al. [25]
	Selenite	392	
	Seleno-L-methionine	130	
	Selenocysteine	83	
Cutex quinquefasciatus (Diptera: Culicidae)	Selenate	11	Jensen et al. [33]
Spodoptera exigua (Lepidoptera: Noctuidae)	Selenate	21	Trumble et al. [21]
	Selenite	9.1	
	Seleno-DL-cystine	15	
	Seleno-DL-methionine	21	
Ceriodaphnia dubia (Daphnid)	Selenate	1.9	Brix et al. [68]
Daphnia pulex (Daphnid)		9.1	
Hyalella azteca (Amphipod)		1.9	
Gammarus lacustris (Amphipod)		3.1	
Gammarus pseudolimnaeus (Amphipod)		1.8	
Chironomus decorus (Diptera: Chironomidae)	Selenate	24	Maier and Knight [62]
	Selenite	48	
	Seleno-DL-methionine	194	
Chironomus riparius (Diptera: Chironomidae)	Selenate	11	Ingersoll et al. [61]
	Seleno-L-methionine	6.9	

[a] LC50s and 95% confidence limits were calculated using log–dose probit analysis in the studies referenced.

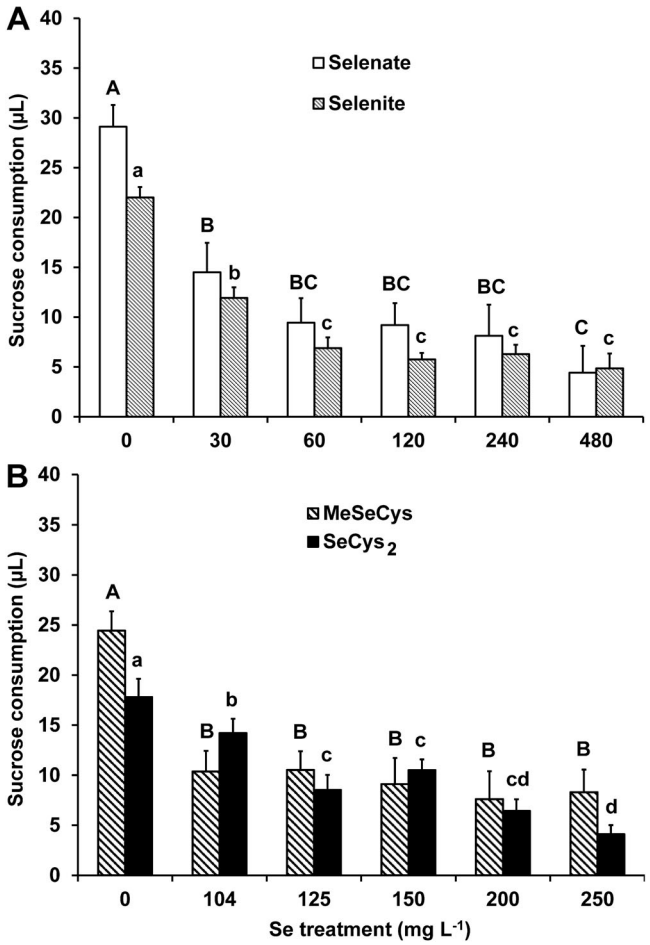

Figure 3. Forager sucrose consumption after dosing with (**A**) selenate or selenite and (**B**) methylselenocysteine (MeSeCys) or selenocystine ($SeCys_2$). Bars (mean ± SE) with the same case and letter are not significantly different within Se species at the $p < 0.05$ level (ANOVA, Tukey's HSD).

succumb to pesticides quickly prevent exposure to the brood, queen, and coworkers [40]. However, honeybee foragers that are not deterred by Se in sucrose solution [22] or in accumulating plants [18] will collect contaminated pollen and nectar and survive the intake of elevated concentrations of Se. When the contaminated floral resources are then distributed to the hive coworkers, Se may be passed on to the brood and have toxic effects on the more susceptible larvae.

All 4 forms of Se had developmental consequences for *A. mellifera* larvae reared in vitro. *Ephydra cinerea* Jones (Diptera: Ephydridae) [41], *Cotesia marginiventris* (Cresson; Hymenoptera: Braconidae) [1], *Heliothis virescens* (Fabricius; Lepidoptera: Noctuidae) [42], *M. scalaris* [25], *Podisus maculiventris* Say (Hemiptera: Pentatomidae) [43], and *S. exigua* [21] also experienced similar sublethal effects when fed Se, including extended development times. In the present study, all 4 Se forms reduced the percentage of larvae reaching the prepupal stage. If honeybee larvae cannot survive to the last larval instar when fed Se, even at these low concentrations, then fewer individuals will pupate into workers, thus reducing the colony numbers.

Selenate, selenite, methylselenocysteine, and selenocystine significantly decreased RGIs. Selenate ingestion reduced the relative growth rate of the herbivorous caterpillar *S. exigua* [21] and the detritivore *M. scalaris* [25], although some of the concentrations tested were higher than those used in the present study. The organic forms of Se (methylselenocysteine, selenocystine) reduced the RGIs as early as 6 d into larval development. Selenium replaces sulfur in amino acids such as cysteine and cystine and can change protein folding, thereby disrupting cell metabolism and causing deformities. The methylation of selenocysteine prevents its misincorporation into proteins in certain accumulating plant species [44]. However, methylselenocysteine is toxic to certain leaf-chewing herbivores [45], but the mechanisms behind the sublethal effects on *A. mellifera* development are unknown, and further studies are warranted.

In a recent review comparing the LD50s of pesticides, *Apis mellifera* was not more susceptible than other insect species [46].

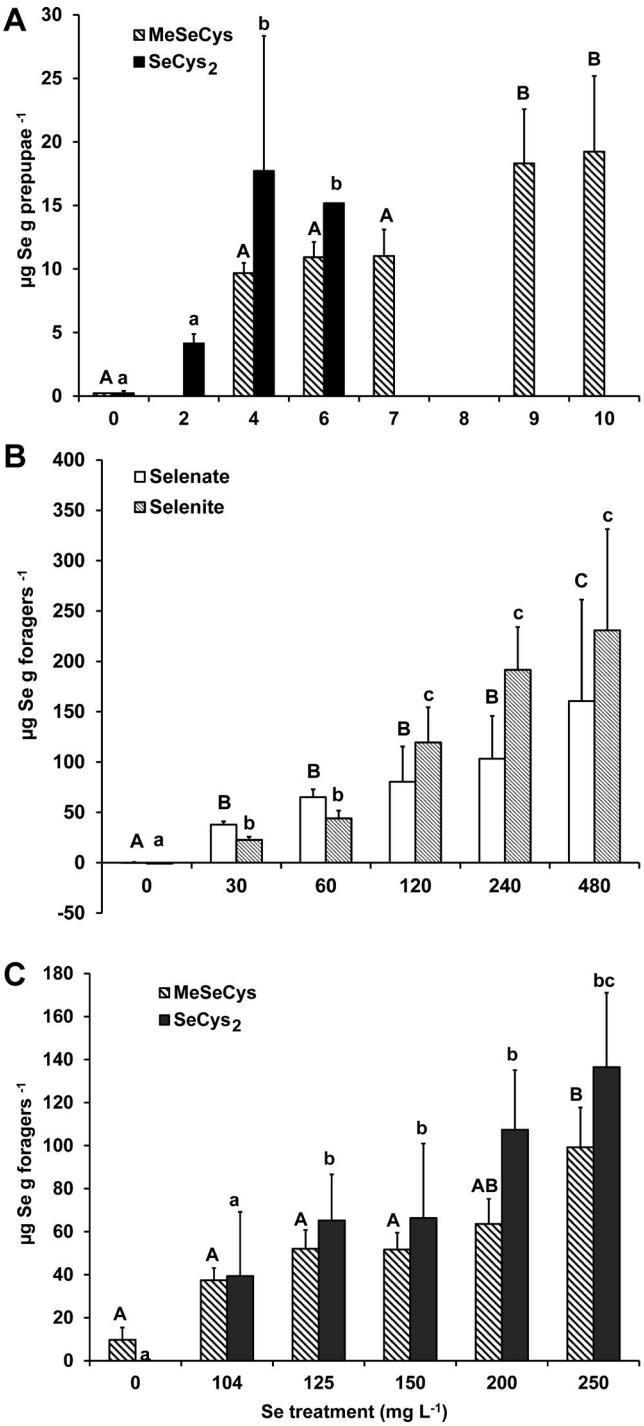

Figure 4. Elemental Se accumulation in prepupae raised on a diet containing (**A**) methylselenocysteine (MeSeCys) or selenocystine ($SeCys_2$). Foragers dosed with (**B**) selenate or selenite and (**C**) MeSeCys or $SeCys_2$ were also analyzed for total elemental Se levels. Bars (mean ± standard error) with the same case and letter are not significantly different within Se species at the $p < 0.05$ level (analysis of variance, Tukey's highly significant difference).

In the present study, however, the LC50s for *A. mellifera* larvae to the metalloid Se were found to be substantially lower than those for other insect species. *Apis mellifera* has fewer detoxification genes compared with other insects [47], which may contribute to the honeybee's sensitivity to the toxicant. In the host *S. exigua* and its parasitoid *C. marginiventris*, tolerance was attributed to methylation and volatilization of Se [1]. In addition, the Brassicaceae herbivore *Plutella xylostella* L. Stanleyi (Lepidoptera: Plutellidae) was highly tolerant to Se; this herbivore may have the ability to keep selenocysteine methylated, thus protecting itself from toxic misincorporation into proteins [48]. In a study conducted in naturally seleniferous areas of Colorado, bumblebees accumulated high concentrations of Se (up to 274 mg Se kg^{-1}), mostly as organic Se [19]. Honeybees accumulated much lower levels of Se overall (up to 15.7 mg Se kg^{-1}) as an organic form of Se throughout their body, including a compound resembling Se-diglutathione. Bumblebees may be more tolerant of Se than honeybees, although the high levels may have been mostly from the pollen remaining on the outside corbicula of the bumblebee. Selenium was also dispersed throughout the pollinators' bodies, indicating no specific site of detoxification. Taken together, the 2 pollinators found foraging on seleniferous plants may have some level of tolerance to the element.

Generally, *A. mellifera* (as well as several other insect species whose genomes have been sequenced) possess no genes for encoding selenoproteins [49] (but see Lobanov et al. [50]) and therefore may not be able to make use of excessive Se ingested in the diet. In the susceptible *A. mellifera* larvae, demethylase enzymes may remove the methyl group from methylselenocysteine, allowing for misincorporation into proteins and altered development. The mechanism for inorganic Se toxicity is not as clear. Some insects, such as *M. domestica* [13] and *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) [51] do not have Se-dependent glutathione peroxidase. Compared with *D. melanogaster* and *Anopheles gambiae* Giles (Diptera: Culicidae), *A. mellifera* has only about half as many antioxidant glutathione-S-transferases [47]. Reactive oxygen species may accumulate and lead to oxidative stress in *A. mellifera* because of excessive levels of selenate being reduced to selenite or selenol [52]. As a result of their lack of detoxification proteins, honeybees must rely on social immunity such as hygienic behaviors or antiseptic products added to pollen and honey to minimize environmental stresses [53]. With only modest amounts of superoxide dismutases, catalases, and peroxidases [54], oxidative stress caused by excessive selenate or selenite may overload *A. mellifera*'s defenses and damage organs such as the peritrophic matrix, which acts as a sink for reactive oxygen species [55].

Adult *A. mellifera* foragers suffered sublethal effects of Se on sucrose consumption in a dose-dependent manner. Certain forms of Se can reduce the bee's responsiveness to sucrose [22]. Several studies have shown that pesticides can increase the bee's sucrose response threshold to the point where it only responds with proboscis extension at high concentrations [56–59]. Foragers dosed with ethanol [60] were less likely to respond to or consume sucrose. The sublethal effects of a toxin on foragers may include a reduced level of arousal or attention to relevant stimuli [60]. Overall the less sucrose the foragers consume after a one-time dose of Se, the less nectar is brought back to the hive to feed the coworkers, brood, and queen, and colony-level consequences may include reductions in food resources such as honey.

The LC50s were approximately 50 times higher for adult foragers compared with larvae, although a similar trend was seen of inorganic forms of Se being more toxic than organic forms at both life stages. *Apis mellifera* foragers had LC50s similar to the pollutant-tolerant *M. scalaris* [25] and midges [61,62]. Certain pesticides can kill foragers quickly, thus protecting the brood, coworkers, and queen by preventing their return to the colony; but when exposed to Se, foragers survive ingestion and may distribute the contaminated nectar to the other more

susceptible members of the hive. Conversely, differences in biomagnification factors between castes have been observed in ants [63], which may be attributable to the dilution effects of trophallaxis. However, Haarmann [64] found no differences in levels of contaminants between honeybee foragers and other castes. Concentrations may be reduced when transferred from caste to caste within the hive, and more studies are needed to closely examine the role of trophallaxis in the biotransfer of contaminants such as Se.

Larvae chronically fed a diet containing selenate accumulated higher levels of Se than those fed organic forms of Se. Adult foragers dosed with inorganic forms of Se accumulated similar levels of Se. Honeybee foragers and larvae did not accumulate significant quantities of methylselenocysteine until fed the higher doses. Although methylation may have prevented excessive accumulation, methylselenocysteine was just as toxic to larvae as selenocystine. Despite the high quantities of Se in forager bodies, the pollutant was still much less toxic to foragers compared with larvae. Larvae are actively growing and developing, and they may be more likely to misincorporate Se-containing amino acids into proteins. Foragers may have a better ability to sequester or detoxify Se than larvae. Older worker bees increase their glutathione S-transferase and mixed-function oxidase activity [65] and may minimize the toxic effects of selenate and selenite despite the high levels of accumulation.

Both adult and juvenile honeybees use pollen as a food source. Pollen is fed to larvae starting on the third day of development. Young workers, especially nurses, need pollen during the first 8 to 10 d for proper postemergence hypopharyngeal gland development [36]. Considering that the highest levels of Se have been found in pollen of certain species of accumulating plants [17,18], both larvae and young workers are at highest risk for ingesting toxic doses through their food. In addition, in polluted environments where Se may not be the only contaminant, co-occurring metals such as mercury can have a synergistic effect and cause higher levels of toxicity than either contaminant alone [33,66]. *Apis mellifera* may be particularly susceptible to synergisms of metal and metalloid contaminants because of its reduced suite of detoxification genes [47]. Similar to pesticide synergies [67], there may be competition for the same detoxification enzymes. Additional experiments examining whole colonies (especially brood responses) at Se-contaminated sites will be required to document potential effects on population dynamics of *A. mellifera*.

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