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**Endosymbiont-mediated resistance to entomotoxic nanoparticles and sex-specific responses in a seed beetle**

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## Abstract

Bacterial symbionts can promote insecticide resistance in their hosts by isolating and degrading insecticidal compounds or altering the expression of host genes. Although *Wolbachia*, a common endosymbiont in arthropods, typically does not influence insecticide resistance, there are cases of increased or decreased susceptibility. Due to the restrictions of applying conventional insecticides in a stored product setting, studies on alternative control methods are needed, including those on entomotoxic nanoparticles (NPs) and the potential for resistance. For pests of stored beans, selenium nanoparticles (SeNPs) are relatively innocuous to the azuki bean beetle, *Callosobruchus chinensis* (L.) (Coleoptera: Chrysomelidae: Bruchinae). Here, we hypothesized that this response is mediated by *Wolbachia*, and we tested this using an isofemale line of *C. chinensis* (infected or uninfected with *Wolbachia*). Our results showed that the lifespan of *Wolbachia*-infected females was not affected by SeNPs, but increasing concentrations of SeNPs still had a negative effect on fecundity; in uninfected females, increasing concentrations of SeNPs significantly decreased both lifespan and fecundity. However, in males, SeNPs enhanced lifespan and decreased the incidence of sexual harassment behavior regardless of infection status (for uninfected males, the duration of harassment behavior also decreased). In the presence of males, 72-h female reproduction increased independent of infection status or SeNP treatment, but egg hatchability was reduced by male presence and SeNPs. This study documents a valuable example of symbiont-mediated resistance to entomotoxic nanoparticles.

**Keywords:** Stored product pests, IPM, nanotechnology, toxicology, microbiome, male harassment

## Key Message

- Insecticide resistance can be mediated by bacterial symbionts
- *Wolbachia* may reduce susceptibility to selenium nanoparticles (SeNPs) in seed beetles
- SeNPs decreased female reproduction, but only uninfected females had a reduced lifespan
- SeNPs increased male lifespan and reduced harassment behavior
- The use of SeNPs can complement other IPM strategies, e.g. the incompatible insect technique (IIT)

## Introduction

Insecticide resistance is a widespread concern in both conventional and integrated pest management as it leads to challenges in pest control and massive economic losses (Pimentel et al. 1992, Sparks and Nauen 2015). Pesticides act as powerful selective pressures, and resistance is typically accomplished by molecular changes at the target site or via altered metabolic processes, usually up-regulation of detoxification enzymes or improved excretion (Sparks and Nauen 2015, IRAC n.d.). Penetration resistance (cuticular thickening or modification; Balabanidou et al. 2018) and behavioral resistance (Sparks et al. 1989; but see Zalucki and Furlong 2017) are also important mechanisms in the evolution of insecticide resistance. However, since the insects themselves are host to a broad diversity of microbiota, it is vital to consider the mechanisms of insecticide resistance (or toxicology) from the broader perspective of the metaorganism (a host and its associated microbiota; see Jaspers et al. 2019).

The insect microbiome is quickly becoming relevant in the context of pest management as more and more cases of symbiont-mediated insecticide resistance are documented (reviewed by Gressel 2017, Pietri and Liang 2018, Blanton and Peterson 2020). For example, *Burkholderia* bacteria in the midgut crypts of some pentatomomorph bugs (Hemiptera: Coreoidea, Lygaeoidea) can degrade fenitrothion (Kikuchi et al. 2012, Ishigami et al. 2021). Similarly, gut bacteria significantly decrease susceptibility to indoxacarb and chlorpyrifos in the tobacco cutworm (*Spodoptera litura* (Fabricius); Lepidoptera: Noctuidae) (Gadad and Vastrad 2016), break down malathion, pirimiphos-methyl, and deltamethrin in grain beetles (Coleoptera: Curculionidae, Laemophloeidae, Bostrichidae) (Wang et al. 2022), mediate resistance to dichlorvos in the cowpea beetle (*Callosobruchus maculatus* (Fabricius); Coleoptera: Chrysomelidae: Bruchinae) (Akami et al. 2019), enhance resistance to chlorpyrifos in the diamondback moth (*Plutella xylostella* (L.); Lepidoptera: Plutellidae) (Xia et al. 2018), and help degrade trichlorfon in the oriental fruit fly (*Bactrocera dorsalis* (Hendel); Diptera: Tephritidae) (Cheng et al. 2017). Interactions with biological products (i.e. *Bacillus thuringiensis*) have also been investigated (Caccia et al. 2016, S. Li et al. 2020).

Bacterial symbionts promote insecticide resistance in their hosts by directly isolating and degrading insecticidal compounds or by indirectly altering the expression of host genes. This latter mechanism was demonstrated for the brown planthopper (*Nilaparvata lugens* (Stål); Hemiptera: Delphacidae), wherein endosymbionts (*Wolbachia* and *Arsenophonus*) and extracellular symbionts (*Acinetobacter*, *Staphylococcus*) confer resistance to chlorpyrifos, imidacloprid, clothianidin, and buprofezin by modulating the expression of cytochrome P450 genes (among other pathways) (Pang et al. 2018, Tang et al. 2021). Of the endosymbiotic bacteria, *Wolbachia* are the most prevalent across arthropod taxa, infecting roughly 60% of species (Hilgenboecker et al. 2008, Weinert et al. 2015). Infections with *Wolbachia* represent a complex system of eco-evolutionary trade-offs between facultative

mutualism and reproductive parasitism (cytoplasmic incompatibility, induced parthenogenesis, feminization, and male-killing) (reviewed by Vavre and Charlat 2012, Zug and Hammerstein 2015, Correa and Ballard 2016). The presence of a *Wolbachia* symbiont typically does not influence insecticide susceptibility; there are some cases of increased susceptibility but very few cases in which *Wolbachia* promotes or mediates resistance (reviewed by Liu and Guo 2019). For example, *Wolbachia* increases resistance to buprofezin in the small brown planthopper (*Laodelphax striatellus* (Fallén); Hemiptera: Delphacidae) (Li et al. 2018, Y. Li et al. 2020) and reduces susceptibility to fipronil and avermectin in the striped rice stemborer (*Chilo suppressalis* (Walker); Lepidoptera: Crambidae) (Lei et al. 2020). Also, *Wolbachia* potentially mediates resistance to fenitrothion and imidacloprid in the tropical bed bug (*Cimex hemipterus* (Fabricius); Hemiptera: Cimicidae) (Soh and Veera Singham 2022) and to organophosphates in the common house mosquito (*Culex pipiens* L.; Diptera: Culicidae) (Berticat et al. 2002).

In addition to insecticide resistance management (see Sparks and Nauen 2015), the choice of insecticide and application strategy is also critical in terms of human health and the environment (Desneux et al. 2007, Damalas and Eleftherohorinos 2011). With these goals in mind, there have been increases in the number of “new generation” insecticides, biorational products, and other biocompatible formulations being tested or incorporated into pest management programs (e.g. Ishaaya et al. 2005; reviewed [in part] by Rosell et al. 2008; but see Goulson 2013, Haddi et al. 2020). Emerging technologies in crop protection and precision agriculture also include the use of nanoparticles (NPs), which either encapsulate (“nanoencapsulate”) a bioactive chemical compound or are toxic to plant-feeding pests on their own (reviewed by Kah and Hofmann 2014, Nuruzzaman et al. 2016, Duhan et al. 2017, Athanassiou et al. 2018). Insecticidal NPs have been based on elemental silicon (and its oxides), silver, gold, and zinc; for example, silica NPs (SiNPs or SiO<sub>2</sub>NPs) can kill pests largely based on their physical mode of action (e.g. damage to cells along digestive tract, abrasion of the outer cuticle, and absorption of cuticular lipids) (reviewed by Benelli 2018). In contrast, the effects of selenium NPs (SeNPs) are less understood, but their mode of action is physiological (although the effects may change if exposure is topical rather than oral): SeNPs slowly release elemental selenium, which reduces growth, developmental rate, and overall survivorship as it accumulates in the Malpighian tubules, midgut, and, potentially, the reproductive tissues (e.g. Hogan and Razniak 1991; reviewed by El-Ramady et al. 2014, Mechora 2019, Garza-García et al. 2022; also see Skalickova et al. 2017). Nevertheless, to the best of our knowledge, there are no published cases of symbiont-mediated resistance to insecticidal nanoparticles.

Ongoing research on the toxicity of SeNPs to bruchine seed beetles (Coleoptera: Chrysomelidae) has identified inconsistent effects across species, with SeNPs significantly decreasing the lifespan of the cowpea beetle (*C. maculatus*) but having little effect on the azuki bean beetle (*C. chinensis*) (Helmy and Tuda et al., in prep). Because *C. chinensis* is naturally infected with the facultative endosymbiont

*Wolbachia* worldwide (whereas *C. maculatus* is not) (Kondo et al. 1999, 2011), we propose that *Wolbachia* may be mediating resistance to SeNPs; the present study will address this hypothesis using an isofemale line of *C. chinensis* either infected with *Wolbachia* or uninfected (treated with tetracycline), and we predict that the reproduction and lifespan of the uninfected line will be negatively affected by SeNPs. However, we do not expect females and males to respond to SeNPs in the same manner due to fundamental differences in physiology (e.g. Yanagi and Miyatake 2003; also see Wagner and Bakare 2016) and both the activity and the tissue/organ-level spatiotemporal dynamics of their *Wolbachia* endosymbionts (Ijichi et al. 2002, Okayama et al. 2016). Because males are more likely to respond positively to low (sublethal) doses of insecticides (see Haddi et al. 2016), we predict a positive sex-specific response in male azuki bean beetles treated with SeNPs. The beneficial or stimulatory effects of low concentrations of insecticides (dose-dependent effects or “hormesis”) can be measured as an increase in adult lifespan or behavioral activity—for male *C. chinensis*, we will assess both lifespan and sexual harassment behavior (Yanagi and Miyatake 2003, Sakurai and Kasuya 2008). Because of the agricultural significance of bruchine seed beetles as stored product pests (and pests of pulse crops) (see Tuda et al. 2006, Tuda 2007, Tuda 2011), and due to the restrictions of applying conventional insecticides in a stored product setting, studies on alternative control methods—including insecticidal NPs (and the potential for resistance)—are essential from an applied perspective of integrated pest management.

## Materials and methods

### *Insect colonies*

Colonies of the azuki bean beetle, *Callosobruchus chinensis* (L.) (Coleoptera: Chrysomelidae: Bruchinae; strain jC from Japan), were maintained in large Petri dishes (9.5 cm in diameter, 4 cm in height) on a single layer of dried azuki beans (*Vigna angularis* (Willdenow) Ohwi & Ohashi cv. Akadaiya (Fabaceae); Daiwa Grain Co., Obihiro, Japan) under standard laboratory conditions (30°C and 60% R.H. with a 16:8 L:D photoperiod). This laboratory colony is naturally infected with two strains of *Wolbachia*: *wBruCon* and *wBruOri* (Kondo et al. 1999). An isofemale line was isolated from the laboratory colony, in which a subset was treated with tetracycline (800 µl of 0.25% tetracycline hydrochloride for six generations) to remove both strains of *Wolbachia*, resulting in two sublines of a single isofemale line: one infected with *Wolbachia* (both strains) and one that is uninfected (confirmed by PCR). Laboratory colonies of both isofemale sublines were established and maintained for use in all of the following experiments. While the use of insect isolines in experimental studies reduces background variation (thus allowing for smaller sample sizes in order to detect an effect), this practice may limit the generalizability of results. Significant effects may be restricted to the isolate under investigation, so future studies of additional isolines might be required to increase confidence in species-level inferences. Also, while tetracycline will negatively

affect other microbial symbionts, significant differences between the untreated and tetracycline-treated isofemale sublines may reasonably be attributed to the absence of *Wolbachia* due to its relative dominance in the azuki bean beetle microbial community (for a similar example with the coffee berry borer (*Hypothenemus hampei* (Ferrari); Coleoptera: Curculionidae: Scolytinae), see Mariño et al 2017).

#### ***Chemical synthesis of SeNPs***

Selenium nanoparticles (SeNPs) were synthesized at room temperature by reducing sodium selenite ( $\text{Na}_2\text{SeO}_3$ ) with ascorbic acid ( $\text{C}_6\text{H}_8\text{O}_6$ ) in the presence of polysorbate 20 as a stabilizing agent (modified from Bartůněk et al. 2015, Vahdati and Tohidi Moghadam 2020; also see Lin et al. 2004, Lin and Wang 2005, Gangadoo et al. 2017). Briefly, a 20-mL stock solution of SeNPs ( $1000 \text{ mg L}^{-1}$ ) was prepared by dissolving 43.8 mg ( $0.253 \text{ mmol}$ )  $\text{Na}_2\text{SeO}_3$  in 17.9 mL ultrapure water (Milli-Q,  $18.2 \text{ M}\Omega\cdot\text{cm}$ ) before adding 100  $\mu\text{L}$  polysorbate 20 and pipette mixing. Next, 2 mL of 0.633 M ascorbic acid ( $1.27 \text{ mmol}$ , for a 5:1 ratio of  $\text{C}_6\text{H}_8\text{O}_6\text{:Na}_2\text{SeO}_3$ ) was added dropwise, and the final solution was vortexed for  $< 5 \text{ s}$  and allowed to sit for 3 min. Finally, the preparation was centrifuged at 12000 rpm for 20 minutes before removing the supernatant and resuspending the SeNPs in ultrapure water. Two concentrations (100 and  $500 \text{ mg L}^{-1}$ ) were made by diluting the stock solution in ultrapure water; a solution consisting solely of ultrapure water ( $0 \text{ mg L}^{-1}$ ) was also included as a control. To characterize the morphology of the SeNPs, a single droplet of SeNPs in solution was air-dried on a lacey carbon film microgrid (NP-C15 [Cu150P], Okenshoji Co., Tokyo, Japan) and examined with TEM (JEM-2100HC, JEOL, Tokyo, Japan) at an accelerating voltage of 200 kV. SeNPs were stored at  $4^\circ\text{C}$  and used within 2 months of synthesis.

#### ***SeNP treatment, infection status, and life history***

The effects of infection status and SeNP treatment on the lifespan and reproduction of male and female beetles were assessed with a laboratory bioassay. Newly emerged ( $< 24 \text{ h}$ ) adult beetles (uninfected or infected with *Wolbachia*) were placed individually in mini-sized Petri dishes (35 mm in diameter, 10 mm in height). A 20- $\mu\text{L}$  droplet of SeNPs (0, 100, or  $500 \text{ mg L}^{-1}$ ) was added to each dish with a micropipette, and each dish was gently shaken to evenly coat all surfaces as well as the beetle. Each beetle was then provided with ten dried azuki beans that were treated in the same manner (but in a separate dish and with a 30- $\mu\text{L}$  droplet; the beans were allowed a short period of time to air-dry before being provided to the beetles). A total of 240 beetles were set up in 12 treatments (sex  $\times$  infection status  $\times$  SeNP concentration), with 20 replicates for each treatment. For the first 24 h of the bioassay, females were paired with males of identical infection status and SeNP treatment (before the males were returned to their respective Petri dishes). To evaluate survivorship, all beetles were monitored daily until death. To record changes in reproduction over time, a subset of 10 females per treatment (infection status  $\times$  SeNP concentration) were

provided with a new set of treated azuki beans every day; the old beans were collected to rear the offspring. The number of hatched eggs and total number of eggs were recorded after 7 days. The entire bioassay was conducted in a growth chamber held at 30°C and 60% R.H. with a 16:8 L:D photoperiod.

### ***Male harassment and reproductive interference***

An etho-assay was performed to test the effects of infection status and SeNP treatment on male sexual harassment. First, newly emerged adults were transferred from laboratory culture into mini-sized Petri dishes, with no more than 5 beetles per dish and sorted by sex and infection status. Since the beetles originated from a mixed-sex laboratory colony, most females had the chance to mate (JRM, personal observation). Next, beetles were treated with 20  $\mu$ L of SeNPs (0 or 500 mg L<sup>-1</sup>). After 24 h, pairs of male and female beetles (of the same infection status and SeNP treatment) were introduced into mini-sized Petri dishes under ambient conditions (25°C, 60% R.H.) and videorecorded from above for 1 h with a digital camera (iPhone 13 Pro, Apple, Cupertino, United States). The video was manually reviewed to quantify the incidence and duration of male harassment behavior as perceived by the female (defined as time spent walking away from a pursuing male). A total of 80 male–female pairs were set up in four treatments (infection status  $\times$  SeNP concentration), with 20 replicates for each treatment.

To complement the etho-assay, a bioassay was also carried out to measure peak female reproduction in the presence and absence of males to determine if any changes in male harassment behavior had a direct effect on female fitness. For this experiment, newly emerged adult females (either *Wolbachia*-infected or uninfected) were transferred from laboratory culture to individual mini-sized Petri dishes and treated with a 20- $\mu$ L droplet of SeNPs (0 or 500 mg L<sup>-1</sup>) and provided with 10 treated azuki beans (as before). For females in the “male present” treatment group, a single newly emerged male of the same infection status and SeNP treatment was added to the female’s Petri dish. The beetles were allowed to reproduce for 72 h (with or without males present), during which time their reproductive output is at its greatest. A total of 96 females were set up in eight treatments (infection status  $\times$  SeNP application  $\times$  male presence), with 12 replicates for each treatment.

### ***Statistics***

The effects of infection status, SeNP concentration, and their interaction on the adult lifespan of male and female azuki bean beetles were tested with separate semiparametric Cox proportional hazards regression models. Because non-ovipositing females (virgin or not) have a longer life expectancy than ovipositing females (Yanagi and Miyatake 2003), females that did not produce viable eggs within the first 24 h were excluded from the analysis (infected, 0 mg L<sup>-1</sup>: -2; 100 mg L<sup>-1</sup>: -2; 500 mg L<sup>-1</sup>: -1) (uninfected, 0 mg L<sup>-1</sup>: -1; 100 mg L<sup>-1</sup>: -1; 500 mg L<sup>-1</sup>: -4).



The lifetime reproduction of females (total number of eggs) was evaluated with a generalized linear model (GLM) using a Poisson distribution and log link function, with infection status, SeNP concentration, and their interaction included as main effects. The daily reproduction of females (eggs per day) was assessed using a generalized linear mixed model (GLMM) with a Poisson distribution and log link function; infection status, SeNP concentration, and the number of days since emergence, as well as all interactions, were included as fixed effects, and the identity of individual females was included as a random effect. Because no viable eggs were produced more than 7 days after adult emergence, the analyses were limited to reproduction on days 0–7. As before, replicates that did not produce viable eggs within the first 24 h were excluded from the analyses (infected, 0 mg L<sup>-1</sup>: -2; 500 mg L<sup>-1</sup>: -1) (uninfected, 0 mg L<sup>-1</sup>: -1; 500 mg L<sup>-1</sup>: -1).

The effects of infection status, SeNP treatment, and their interaction on male harassment behavior were divided into two components: (i) the incidence of harassment (the presence or absence of any harassment behavior within the 1-h observation period) and (ii) the duration of harassment behaviors (the time, in seconds, of each occurrence of harassment behavior between a male–female pair during the 1-h observation period). The incidence of harassment was analyzed using a logistic regression model. The duration of male harassment was analyzed with a semiparametric Cox proportional hazards regression with male–female pair ID incorporated as a random effect (frailty).

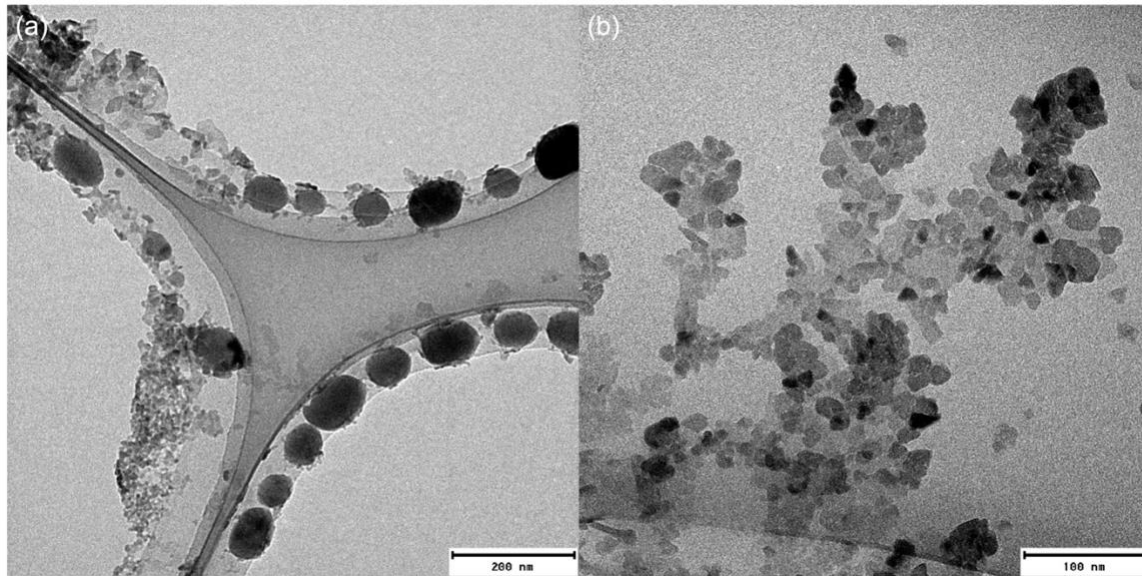
A multiple regression model was used to test the effects of infection status, SeNP treatment (0 mg L<sup>-1</sup> or 500 mg L<sup>-1</sup>), and the presence or absence of males (and all two-way interactions) on the total number of eggs laid per female over the 72-h period. Pairwise comparisons were made with FDR-corrected *p*-values. The same procedure was used to assess the effects of infection status, SeNP treatment, and the presence or absence of males (and all two-way interactions) on the logit-transformed proportion of hatched eggs produced per female over the 72-h period (see Warton and Hui 2011). Replicates that did not produce eggs (likely because the female did not mate prior to the study period) or in which the female (or male, if present) died within the 72-h period were excluded from the analysis (infected, 0 mg L<sup>-1</sup>, male absent: -2; male present: -1) (infected, 500 mg L<sup>-1</sup>, male absent: -4) (uninfected, 500 mg L<sup>-1</sup>, male absent: -2; male present: -1).

All statistical analyses were performed in R version 4.2.0 (The R Foundation for Statistical Computing 2022). The survivorship analysis also used the *survival*, *emmeans*, and *multcomp* packages; the fertility analysis used *lme4*, *optimx*, *afex*, *multcomp*, and *emmeans*; the behavioral analysis used *survival*, *car*, and *coxed*; and the reproductive interference analysis used *car* and *agricolae*. Figures were prepared in base R with the addition of the *yarr* package (for transparent colors).

## Results

### SeNP characterization

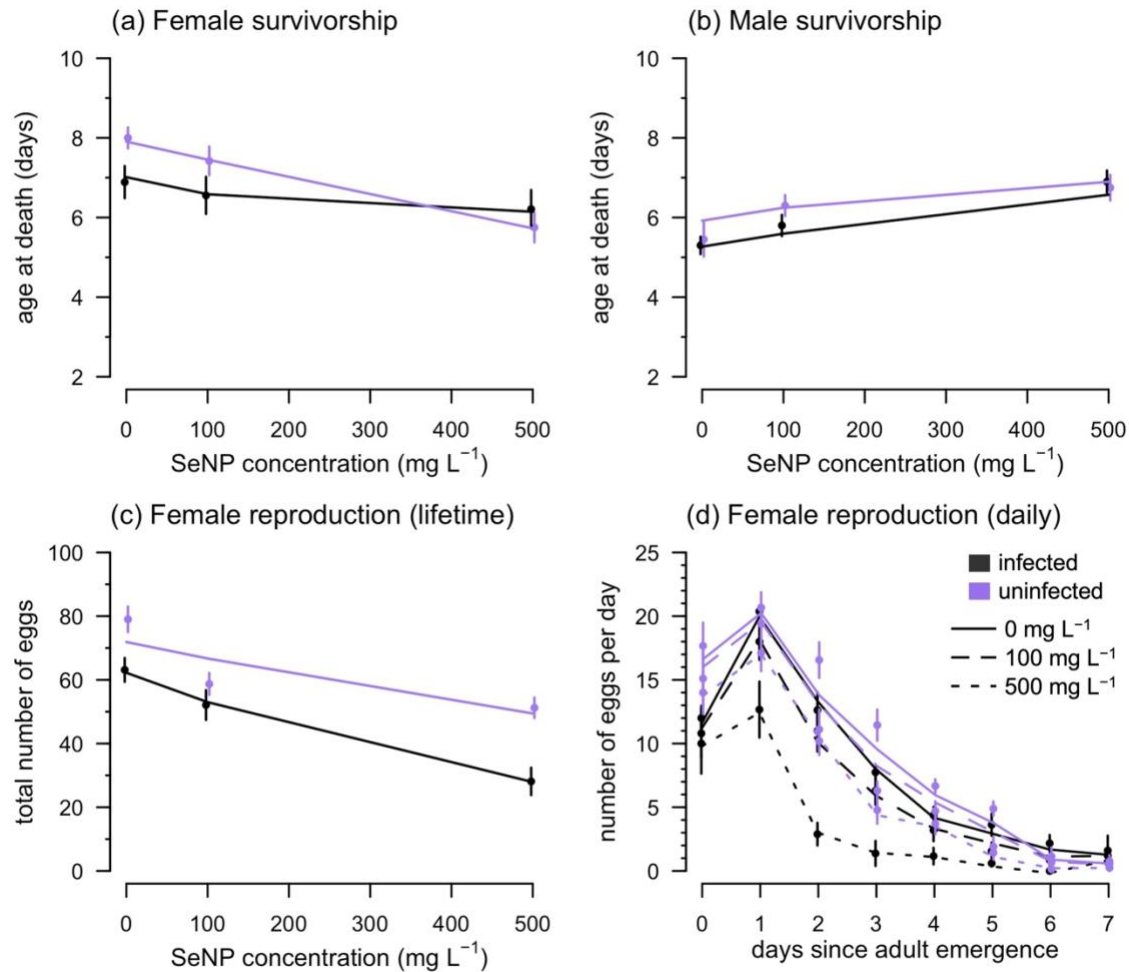
Reducing sodium selenite with ascorbic acid and stabilizing with polysorbate 20 yielded amorphous selenium nanoparticles (SeNPs) in two size classes (approximately 5–10 and 60–100 nm in diameter), the smaller of which were similar to the nanoparticulate debris produced by laser ablation and, likely during the desiccation processes for imaging, often self-polymerized into filamentous structures (Fig. 1).



**Fig. 1** Chemically synthesized selenium nanoparticles on a lacey carbon film microgrid. **a** Large and small amorphous SeNPs and **b** small, self-polymerizing SeNPs

### SeNP treatment, infection status, and life history

The adult lifespan of uninfected female azuki bean beetles was negatively affected by increasing SeNP concentration, whereas that of *Wolbachia*-infected females was unaffected by SeNP concentration (infection status  $\times$  SeNP concentration; Fig. 2a and Table 1). Also, while uninfected females tended to live longer than *Wolbachia*-infected females, this difference was not statistically significant ( $p = 0.084$ ; Fig. 2a and Table 1). The adult lifespan of male azuki bean beetles was greater for uninfected individuals than *Wolbachia*-infected individuals and was enhanced with increasing SeNP concentration (Fig. 2b and Table 1).



**Fig. 2** Effects of SeNPs and *Wolbachia* infection on the lifespan and reproduction of adult azuki bean beetles. **a** Female survivorship. **b** Male survivorship. **c** Female lifetime reproduction (total number of eggs per female). **d** Daily reproduction (number of eggs laid per day). In all panels: points: means  $\pm$  SE; lines: model predictions

**Table 1** Lifespan of female and male azuki bean beetles: analysis of deviance tables for the Cox proportional hazards models

	Predictor	$\chi^2$	df	<i>p</i> -value
Female	infection status	2.98	1	0.084
	SeNP concentration	1.28	1	0.258
	infection status $\times$ SeNP conc.	4.80	1	<b>0.028</b>
Male	infection status	4.64	1	<b>0.031</b>
	SeNP concentration	21.53	1	<b>&lt; 0.001</b>
	infection status $\times$ SeNP conc.	1.60	1	0.206

The lifetime reproduction of female azuki bean beetles was lower for *Wolbachia*-infected beetles than for uninfected beetles and decreased with increasing SeNP concentration (Fig. 2c and Table 2). Additionally, while SeNP concentration led to a sharper decline in the reproduction of *Wolbachia*-infected females than for uninfected females, the rate of decline decreased as SeNP concentration continued to increase (infection status  $\times$  SeNP concentration; Fig. 2c and Table 2). The daily reproduction of females decreased with increasing SeNP concentration and over time (after peaking on day 1), and while that of uninfected females tended to be greater than that of infected females, this trend was not statistically significant (Fig. 2d and Table 2). Increasing SeNP concentration reduced the daily reproduction of infected females more dramatically than uninfected females (infection status  $\times$  SeNP concentration; Fig. 2d and Table 2). Although marginally significant ( $p = 0.052$ ), increasing SeNP concentration tended to decrease daily reproduction from day 0, with the magnitude of this effect changing over time; in general, the effects initially increased before decreasing with time (SeNP concentration  $\times$  day; Fig. 2d and Table 2). On day 0, the effect of increasing SeNP concentration on the daily reproduction of infected females was less pronounced than for uninfected females, and the magnitude of the effect of increasing SeNP concentration increased for infected individuals on days 1 and 2, whereas the magnitude of the effect of increasing SeNP concentration remained relatively constant for uninfected females, with a relatively small peak on day 3 (infection status  $\times$  SeNP concentration  $\times$  day; Fig. 2d and Table 2).

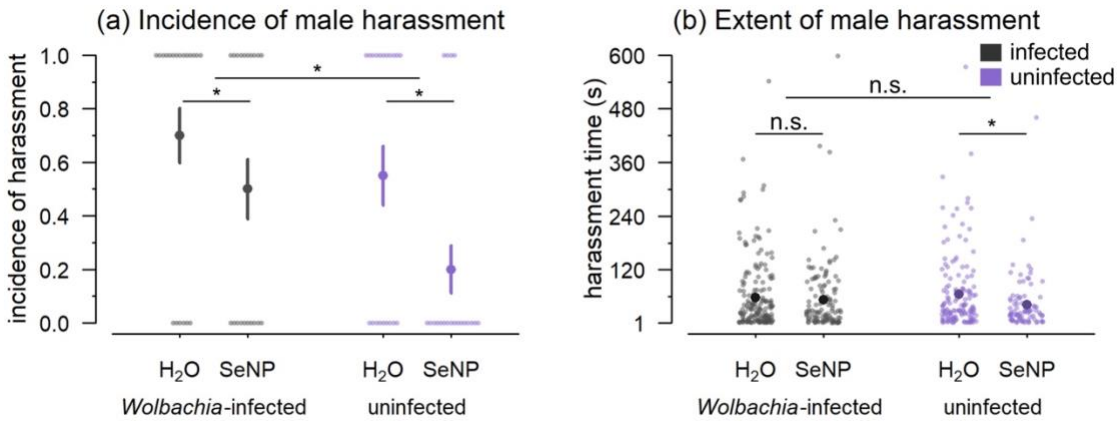
**Table 2** Reproduction of female azuki bean beetles: analysis of deviance tables for the GLM of total reproduction and the GLMM of daily reproduction

Predictor	Total reproduction			Daily reproduction		
	$\chi^2$	df	$p$ -value	$\chi^2$	df	$p$ -value
infection status	9.58	1	<b>0.002</b>	0.19	1	0.664
SeNP concentration	124.84	1	<b>&lt; 0.001</b>	30.94	1	<b>&lt; 0.001</b>
day	—	—	—	316.81	7	<b>&lt; 0.001</b>
infection status $\times$ SeNP conc.	19.86	1	<b>&lt; 0.001</b>	4.74	1	<b>0.029</b>
infection status $\times$ day	—	—	—	13.93	7	<b>0.052</b>
SeNP conc. $\times$ day	—	—	—	36.87	7	<b>&lt; 0.001</b>
infection status $\times$ SeNP conc. $\times$ day	—	—	—	15.06	7	<b>0.035</b>

### ***Male harassment and reproductive interference***

The incidence of male harassment behavior was lower in uninfected male–female pairs than in *Wolbachia*-infected pairs, and was also lower when pairs were treated with 500 mg L<sup>-1</sup> SeNPs (Fig. 3a and Table 3). The incidence of male harassment was not affected by an interaction between infection status and SeNP treatment (Table 3). For male–female pairs in which harassment occurred, the duration

of male harassment behavior was unaffected by infection status, marginally decreased with SeNP treatment, and significantly affected by an interaction between infection status and SeNP treatment—SeNPs significantly decreased harassment behavior, but only in uninfected male–female pairs (Fig. 3b and Table 4). The random effect (frailty) of male–female pair ID was also significant, indicating that the typical duration of harassment behaviors varied from one pair to the next (Table 4).



**Fig. 3** Effects of infection status and SeNPs (0 or 500 mg L<sup>-1</sup>) on male harassment behavior in azuki bean beetles. **a** Incidence of male harassment behavior during the 1-h observation period; points with solid bars indicate the predicted values ± SE from the logistic regression model; asterisks indicate significant differences between SeNP treatments and infection status. **b** Duration of male harassment over the 1-h observation period; dark circles plot the mean predicted values ± SE from the Cox proportional hazards model (SE are not visible because they are smaller than the circles); asterisks indicate significant differences between SeNP treatments and between infection statuses

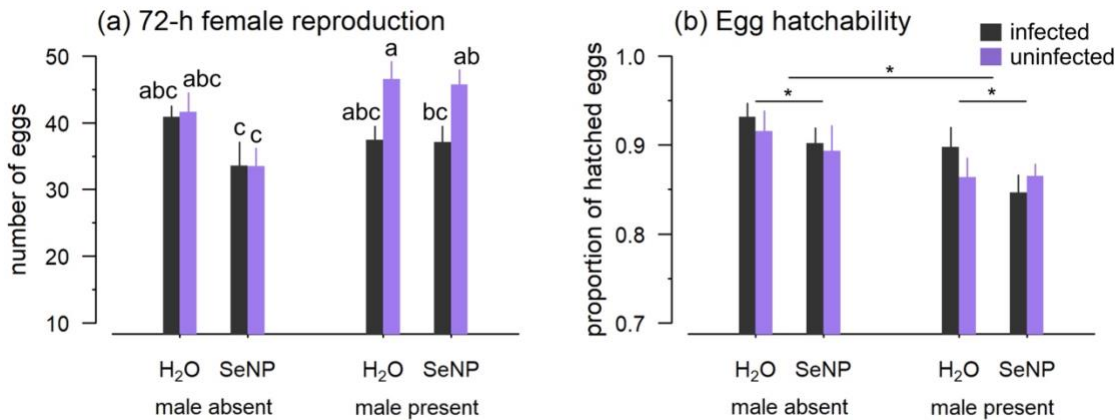
**Table 3** Incidence of male harassment behavior in azuki bean beetles: analysis of deviance table for the logistic model

Predictor	Likelihood-ratio $\chi^2$	df	<i>p</i> -value
infection status	4.44	1	<b>0.035</b>
SeNP treatment	6.48	1	<b>0.011</b>
infection status × SeNP trt.	0.58	1	0.447

**Table 4** Duration of male harassment behavior in azuki bean beetles: analysis of deviance table for the Cox proportional hazards model

Predictor	$\chi^2$	df	p-value
infection status	0.00	1	0.950
SeNP treatment	3.35	1	0.067
male–female pair ID	14.61	1	< 0.001
infection status $\times$ SeNP trt.	94.16	19.67	< 0.001

Female reproduction over a 72-h period was lower when individuals were infected with *Wolbachia* or treated with SeNPs (Fig. 4a and Table 5); SeNPs also decreased egg hatchability (Fig. 4b and Table 6). When males were present, female reproduction increased (Fig. 4a) while egg hatchability decreased (Fig. 4b) (Table 6). There were also significant interactions between male presence and both infection status and SeNP treatment on female reproduction, with male presence having a stronger positive effect on the reproduction of uninfected females and SeNPs enhancing the positive effects of male presence (Fig. 4a, Table 5); there were no other significant interactions for female reproduction or hatchability. However, the *post hoc* analysis of 72-h reproduction was unable to resolve many differences among the eight groups; water-treated uninfected females in the presence of males laid more eggs than SeNP-treated infected females in the absence of males, and both water- and SeNP-treated uninfected females in the presence of males laid more eggs than SeNP-treated infected or uninfected females in the absence of males (Fig. 4a).



**Fig 4** Effects of male presence, SeNPs (0 or 500 mg L<sup>-1</sup>), and infection status on the reproduction of female azuki bean beetles. **a** Mean ( $\pm$  SE) number of eggs laid per female over 72 h in the absence and presence of males; shared letters indicate no significant differences. **b** Proportion of hatched eggs; asterisks indicate significant differences between SeNP concentrations and for male presence

**Table 5** The 72-h reproduction of female azuki bean beetles: analysis of deviance table for the multiple regression model

Predictor	Likelihood-ratio $\chi^2$	df	<i>p</i> -value
infection status	7.32	1	<b>0.007</b>
SeNP treatment	4.79	1	<b>0.029</b>
male presence	5.82	1	<b>0.016</b>
infection status $\times$ SeNP trt.	0.03	1	0.864
infection status $\times$ male presence	5.58	1	<b>0.018</b>
SeNP trt. $\times$ male presence	3.85	1	<b>0.0497</b>

**Table 6** Proportion of hatched azuki bean beetle eggs: analysis of deviance table for the multiple regression model of the logit-transformed proportion of hatched eggs

Predictor	Likelihood-ratio $\chi^2$	df	<i>p</i> -value
infection status	0.14	1	0.709
SeNP treatment	4.79	1	<b>0.029</b>
male presence	11.20	1	<b>&lt; 0.001</b>
infection status $\times$ SeNP trt.	1.03	1	0.309
infection status $\times$ male presence	0.31	1	0.577
SeNP trt. $\times$ male presence	0.03	1	0.856

## Discussion

Symbiont-mediated insecticide resistance is a significant issue that highlights the need to study pest biology from a metaorganismal perspective, especially within the context of next-generation insecticides, biorational products, and nanotechnology. The endosymbiont *Wolbachia* has been found to confer insecticide resistance in just a few cases (reviewed by Liu and Guo 2019), and our results offer a novel example of sex-specific *Wolbachia*-mediated resistance to entomotoxic nanoparticles in the azuki bean beetle, *Callosobruchus chinensis*. However, since *Wolbachia* only mediated resistance to selenium nanoparticles (SeNPs) in female beetles (in terms of lifespan, but not fertility), whereas males exhibited SeNP-induced hormesis (but a reduction in sexual harassment behavior) regardless of infection status, these results have unique implications for incorporating SeNPs into pest management programs.

Little is known about the insecticidal mode of action of SeNPs when absorbed through the cuticle. Unlike SiNPs, the mode of action is primarily physiological rather than mechanical (reviewed by Mechora 2019). SeNPs slowly release elemental selenium, and selenium reduces the growth, developmental rate, and overall survivorship of pest insects; for example, the beet armyworm (*Spodoptera exigua* (Hübner); Lepidoptera: Noctuidae) is differentially affected by sodium selenate, sodium selenite, selenocysteine, and selenomethionine (Trumble et al. 1998), and larvae of the mealworm beetle (*Tenebrio molitor* L.; Coleoptera: Tenebrionidae), reared in a medium containing sodium selenite,

have reduced survivorship as selenium primarily accumulates in the Malpighian tubules (Hogan and Razniak 1991). In the house fly (*Musca domestica* L.; Diptera: Muscidae), the toxicity of selenium is reduced due to accumulation in the midgut, where cells sequester selenium in lysosomes (Simmons et al. 1988). A related study of the rice meal moth (*Corcyra cephalonica* (Stainton); Lepidoptera: Pyralidae) found that selenium accumulates in the mitochondria (also noted by Simmons et al. 1988, and mostly in the mitochondrial membrane), which may actually have positive effects on mitochondrial energetics due to the presence of an unknown selenocysteine-containing protein or selenoenzyme (Lalitha et al. 1994).

Based on our hypothesis of *Wolbachia*-mediated resistance to SeNPs in the azuki bean beetle, our results partially supported the prediction that uninfected female beetles would be negatively affected by SeNPs via a reduction in adult lifespan and fecundity. Increasing concentrations of SeNPs decreased the lifespan of uninfected female beetles, whereas the lifespan of *Wolbachia*-infected females was not affected. The reproduction of infected and uninfected beetles generally decreased with increasing SeNP concentration, and these effects were less pronounced for *Wolbachia*-infected females than for uninfected females for the first 24 h post-exposure; however, over time, SeNPs reduced the total reproduction of infected females more than that of uninfected females. Because of the close association of *Wolbachia* with host reproductive tissues (Ijichi et al. 2002), this suggests the existence of a set of life history trade-offs with respect to endosymbiont-mediated resistance to SeNPs. On the contrary, in males, increasing concentrations of SeNPs enhanced lifespan regardless of infection status, although this does not necessarily contradict our prediction—we did not expect SeNPs to have the same effects for beetles of both sexes due to fundamental differences in physiology and the dynamics of infection (Yanagi and Miyatake 2003, Wagner and Bakare 2016, Ijichi et al. 2002). In the small brown planthopper (*Laodelphax striatellus*), *Wolbachia* may not only enhance the immune system by altering host gene expression, but, in males, also increases the expression of genes related to the metabolism of selenocompounds (Liu et al. 2019). Interestingly, in the Gulf Coast tick (*Amblyomma maculatum* Koch; Arachnida: Ixodida: Ixodidae), the selenoprotein thioredoxin reductase (TrxR) plays an important role in structuring the bacterial community in the microbiome (Budachetri and Karim 2015). Future studies should explore the physiological mechanisms by which *Wolbachia* mediates the response to SeNPs in seed beetles.

Compared to females, males are more likely to respond positively to sublethal doses of insecticides when exposed to these environmental stressors early in life (see Haddi et al. 2016), thus we predicted an increase in the lifespan and behavioral activity of males in response to SeNPs (hormesis: the beneficial or stimulatory effects of sublethal concentrations of insecticides). Supporting our hypothesis, increasing concentrations of SeNPs enhanced male lifespan, but the lack of a *Wolbachia*-mediated effect on male lifespan may be due to the relative non-effect of selective pressures (for detoxifying substances that are harmful to their host) since males are typically an evolutionary “dead end.” However, we did not



expect to find that the incidence of sexual harassment behavior decreased when males were treated with SeNPs. While the measured response variable was the amount of time that females spent avoiding male advances, the lack of interactions appeared to be due to a lack of male harassment (there was no need for females to avoid males, as opposed to an SeNP-triggered change in how females responded to harassment). Also of note, uninfected beetles exhibited lower incidences of harassment, but this could be anticipated since *Wolbachia* increases the locomotory activity of *C. chinensis* (Okayama et al. 2016). SeNPs also led to a marginally significant decrease in the duration of male harassment behaviors as well as a significant decrease in duration for uninfected male–female pairs, which suggests that *Wolbachia* may also be modulating the response to SeNPs in males, possibly via altered cellular energetics or metabolic function.

In a related species, the lifetime reproductive effort in males is equal to that of females (Wagner and Bakare 2016), so a reduction in male sexual behaviors may redirect significantly more resources towards male survival. Yet this overall decrease in behavioral activity raises the question of whether SeNPs truly have a stimulatory effect on male *C. chinensis*, so it may not be entirely accurate to describe this response as “hormesis” (it is also currently unknown at what dose SeNPs would be lethal to males, although, in general, the toxicity of NPs is not comparable to that of conventional insecticides—SeNPs might be better described as “entomotoxic” as opposed to “insecticidal” *sensu stricto*; e.g. Debnath et al. 2011). It may also be that SeNPs help reduce sexual conflict by calming males after being rejected by a partner (thus reducing the incidence or recurrence of harassment behavior). Regardless, the presence of males tended to increase female reproduction (especially when treated with SeNPs), which may indicate a shift in the risk-benefit dynamics associated with a lower frequency of remating in nutrient-poor conditions (such as a stored product setting): male harassment and injuries sustained during copulation are minimized while nuptial gifts (water or nutrients) are still available (Miyatake and Matsumura 2004, Harano et al. 2006, Rönn et al. 2006, Sakurai and Kasuya 2008, Harano 2015; for an example with singly mated *C. chinensis*, see Yanagi and Miyatake 2003; for examples involving *C. maculatus*, see Fox 1993, Arnqvist et al. 2005, Gay et al. 2009, den Hollander and Gwynne 2009).

However, it may be equally likely that the presence of males promotes female reproduction in some other way because, for the “average” female, remating is expected to decrease fecundity in *C. chinensis* (Harano et al. 2006). The stress of male presence, independent of the magnitude of harassment behavior, could increase the oviposition rate of the female in order to compensate (if females respond to SeNPs as a source of environmental stress as well, this might explain the additive effects of both treatments). Regardless, in our study, even though female reproduction increased in the presence of males, female fitness was still lower due to reduced egg hatchability; since there was no statistical interaction between male presence and SeNP treatment on egg hatchability, this suggests that other

aspects of male activity may be unaffected if not stimulated by SeNPs (i.e. walking, trampling eggs—see Shimada and Tuda 1996, Fujii 2009).

#### ***SeNPs and the integrated pest management of seed beetles***

Entomotoxic nanoparticles are interesting examples of an emerging technology in precision agriculture with applications in insecticide resistance management (reviewed by Kah and Hofmann 2014, Duhan et al. 2017, Athanassiou et al. 2018; also see Sparks and Nauen 2015). A novel way of incorporating SeNPs into integrated pest management (IPM) programs might combine targeted SeNP applications with the release of *Wolbachia*-infected males, using the incompatible insect technique (IIT) to induce cytoplasmic incompatibility (CI) in an uninfected pest population (Lees et al. 2015). Because of the high prevalence of *Wolbachia* in *C. chinensis*, the pest population would first need to be treated with antibiotics (but see Khachatourians 1998, Witte 1998, Smith et al. 2002). At this point, because uninfected females have a higher fitness than *Wolbachia*-infected females, SeNPs could be applied to counteract this effect. Next, *Wolbachia*-infected males can be released to induce CI (Brelsfoard and Dobson 2009; for an example with *Aedes* mosquitoes (Diptera: Culicidae), see O'Connor et al. 2012, Crawford et al. 2020 [but see Bouyer et al. 2022]). A lower concentration of SeNPs can be applied at this stage to enhance male lifespan, increasing the likelihood that the released males mate with as many different females as possible while minimizing behavioral changes (to keep levels of harassment high). Another behavioral advantage is that infected males have higher levels of locomotory activity than uninfected males, resulting in more mating opportunities (Nakayama and Miyatake 2010, Okayama et al. 2016); again, while SeNPs appear to reduce overall activity in males, this reduction does not fall below the activity of uninfected males. However, the effects of SeNPs on copulatory behavior, ejaculate volume/composition, and sperm quality are currently unknown, but would be relevant to the success of released males (Okayama et al. 2016).

#### ***Conclusion***

Our study offers a unique example of *Wolbachia*-mediated resistance to SeNPs in female seed beetles, with differential effects on male survivorship and behavior. Compared to other forms of selenium, SeNPs are less hazardous towards humans and other non-target organisms (Wang et al. 2007, Zhang et al. 2008; for biomedical applications, see Ramya et al. 2015, Vinković Vrček 2018). But as an emerging technology, there are a number of unknowns regarding both the biological and ecotoxicity of SeNPs. In fact, the growing use of nanomaterials does raise a number of concerns, from dose-dependent phytotoxicity (e.g. SiO<sub>2</sub>NPs; Thabet et al. 2019) to effects on human health and the environment (Hansen et al. 2008, Wang et al. 2010; SeNPs: Kumar et al. 2018; reviewed by El-Ramady et al. 2014; for a discussion on regulations, see Chau et al. 2007). Regardless, the incorporation of nanoparticles into

management programs (and with a deeper understanding of the role of the insect microbiome) may prove to be a valuable complementary technique in the fight against pests.

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## **Declarations**

### ***Ethical approval***

The authors affirm that the present study was carried out in adherence to all applicable ethical standards.

### ***Competing interests***

The authors declare no conflicts of interest or competing interests.

### ***Authors' contributions***

All authors contributed to the study conception and design. Material preparation and data collection were performed by JRM. All authors participated in data analysis. The first draft of the manuscript was written by JRM, and all authors commented on subsequent versions of the manuscript. All authors read and approved the final manuscript.

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### ***Availability of data and materials***

The datasets associated with the current study are available from the primary corresponding author on reasonable request.

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