

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

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

3

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19 **Abstract**

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

37

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

39

40 **Key Message**

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

## 47 **Introduction**

48 Insecticide resistance is a widespread concern in both conventional and integrated pest management as it  
49 leads to challenges in pest control and massive economic losses (Pimentel et al. 1992, Sparks and Nauen  
50 2015). Pesticides act as powerful selective pressures, and resistance is typically accomplished by  
51 molecular changes at the target site or via altered metabolic processes, usually up-regulation of  
52 detoxification enzymes or improved excretion (Sparks and Nauen 2015, IRAC n.d.). Penetration  
53 resistance (cuticular thickening or modification; Balabanidou et al. 2018) and behavioral resistance  
54 (Sparks et al. 1989; but see Zalucki and Furlong 2017) are also important mechanisms in the evolution of  
55 insecticide resistance. However, since the insects themselves are host to a broad diversity of microbiota, it  
56 is vital to consider the mechanisms of insecticide resistance (or toxicology) from the broader perspective  
57 of the metaorganism (a host and its associated microbiota; see Jaspers et al. 2019).

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

72         Bacterial symbionts promote insecticide resistance in their hosts by directly isolating and  
73 degrading insecticidal compounds or by indirectly altering the expression of host genes. This latter  
74 mechanism was demonstrated for the brown planthopper (*Nilaparvata lugens* (Stål); Hemiptera:  
75 Delphacidae), wherein endosymbionts (*Wolbachia* and *Arsenophonus*) and extracellular symbionts  
76 (*Acinetobacter*, *Staphylococcus*) confer resistance to chlorpyrifos, imidacloprid, clothianidin, and  
77 buprofezin by modulating the expression of cytochrome P450 genes (among other pathways) (Pang et al.  
78 2018, Tang et al. 2021). Of the endosymbiotic bacteria, *Wolbachia* are the most prevalent across  
79 arthropod taxa, infecting roughly 60% of species (Hilgenboecker et al. 2008, Weinert et al. 2015).  
80 Infections with *Wolbachia* represent a complex system of eco-evolutionary trade-offs between facultative

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

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

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

115 *Wolbachia* worldwide (whereas *C. maculatus* is not) (Kondo et al. 1999, 2011), we propose that  
116 *Wolbachia* may be mediating resistance to SeNPs; the present study will address this hypothesis using an  
117 isofemale line of *C. chinensis* either infected with *Wolbachia* or uninfected (treated with tetracycline), and  
118 we predict that the reproduction and lifespan of the uninfected line will be negatively affected by SeNPs.  
119 However, we do not expect females and males to respond to SeNPs in the same manner due to  
120 fundamental differences in physiology (e.g. Yanagi and Miyatake 2003; also see Wagner and Bakare  
121 2016) and both the activity and the tissue/organ-level spatiotemporal dynamics of their *Wolbachia*  
122 endosymbionts (Ijichi et al. 2002, Okayama et al. 2016). Because males are more likely to respond  
123 positively to low (sublethal) doses of insecticides (see Haddi et al. 2016), we predict a positive sex-  
124 specific response in male azuki bean beetles treated with SeNPs. The beneficial or stimulatory effects of  
125 low concentrations of insecticides (dose-dependent effects or “hormesis”) can be measured as an increase  
126 in adult lifespan or behavioral activity—for male *C. chinensis*, we will assess both lifespan and sexual  
127 harassment behavior (Yanagi and Miyatake 2003, Sakurai and Kasuya 2008). Because of the agricultural  
128 significance of bruchine seed beetles as stored product pests (and pests of pulse crops) (see Tuda et al.  
129 2006, Tuda 2007, Tuda 2011), and due to the restrictions of applying conventional insecticides in a stored  
130 product setting, studies on alternative control methods—including insecticidal NPs (and the potential for  
131 resistance)—are essential from an applied perspective of integrated pest management.

132

## 133 **Materials and methods**

### 134 *Insect colonies*

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

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

153

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

155 Selenium nanoparticles (SeNPs) were synthesized at room temperature by reducing sodium selenite  
156 ( $\text{Na}_2\text{SeO}_3$ ) with ascorbic acid ( $\text{C}_6\text{H}_8\text{O}_6$ ) in the presence of polysorbate 20 as a stabilizing agent (modified  
157 from Bartůněk et al. 2015, Vahdati and Tohidi Moghadam 2020; also see Lin et al. 2004, Lin and Wang  
158 2005, Gangadoo et al. 2017). Briefly, a 20-mL stock solution of SeNPs ( $1000 \text{ mg L}^{-1}$ ) was prepared by  
159 dissolving 43.8 mg (0.253 mmol)  $\text{Na}_2\text{SeO}_3$  in 17.9 mL ultrapure water (Milli-Q,  $18.2 \text{ M}\Omega\cdot\text{cm}$ ) before  
160 adding 100  $\mu\text{L}$  polysorbate 20 and pipette mixing. Next, 2 mL of 0.633 M ascorbic acid (1.27 mmol, for a  
161 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  
162 allowed to sit for 3 min. Finally, the preparation was centrifuged at 12000 rpm for 20 minutes before  
163 removing the supernatant and resuspending the SeNPs in ultrapure water. Two concentrations (100 and  
164  $500 \text{ mg L}^{-1}$ ) were made by diluting the stock solution in ultrapure water; a solution consisting solely of  
165 ultrapure water ( $0 \text{ mg L}^{-1}$ ) was also included as a control. To characterize the morphology of the SeNPs, a  
166 single droplet of SeNPs in solution was air-dried on a lacey carbon film microgrid (NP-C15 [Cu150P],  
167 Okenshoji Co., Tokyo, Japan) and examined with TEM (JEM-2100HC, JEOL, Tokyo, Japan) at an  
168 accelerating voltage of 200 kV. SeNPs were stored at  $4^\circ\text{C}$  and used within 2 months of synthesis.

169

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

171 The effects of infection status and SeNP treatment on the lifespan and reproduction of male and female  
172 beetles were assessed with a laboratory bioassay. Newly emerged ( $< 24 \text{ h}$ ) adult beetles (uninfected or  
173 infected with *Wolbachia*) were placed individually in mini-sized Petri dishes (35 mm in diameter, 10 mm  
174 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,  
175 and each dish was gently shaken to evenly coat all surfaces as well as the beetle. Each beetle was then  
176 provided with ten dried azuki beans that were treated in the same manner (but in a separate dish and with  
177 a 30- $\mu\text{L}$  droplet; the beans were allowed a short period of time to air-dry before being provided to the  
178 beetles). A total of 240 beetles were set up in 12 treatments (sex  $\times$  infection status  $\times$  SeNP concentration),  
179 with 20 replicates for each treatment. For the first 24 h of the bioassay, females were paired with males of  
180 identical infection status and SeNP treatment (before the males were returned to their respective Petri  
181 dishes). To evaluate survivorship, all beetles were monitored daily until death. To record changes in  
182 reproduction over time, a subset of 10 females per treatment (infection status  $\times$  SeNP concentration) were

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

186

### 187 *Male harassment and reproductive interference*

188 An etho-assay was performed to test the effects of infection status and SeNP treatment on male sexual  
189 harassment. First, newly emerged adults were transferred from laboratory culture into mini-sized Petri  
190 dishes, with no more than 5 beetles per dish and sorted by sex and infection status. Since the beetles  
191 originated from a mixed-sex laboratory colony, most females had the chance to mate (JRM, personal  
192 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  
193 and female beetles (of the same infection status and SeNP treatment) were introduced into mini-sized  
194 Petri dishes under ambient conditions (25°C, 60% R.H.) and videorecorded from above for 1 h with a  
195 digital camera (iPhone 13 Pro, Apple, Cupertino, United States). The video was manually reviewed to  
196 quantify the incidence and duration of male harassment behavior as perceived by the female (defined as  
197 time spent walking away from a pursuing male). A total of 80 male–female pairs were set up in four  
198 treatments (infection status  $\times$  SeNP concentration), with 20 replicates for each treatment.

199 To complement the etho-assay, a bioassay was also carried out to measure peak female  
200 reproduction in the presence and absence of males to determine if any changes in male harassment  
201 behavior had a direct effect on female fitness. For this experiment, newly emerged adult females (either  
202 *Wolbachia*-infected or uninfected) were transferred from laboratory culture to individual mini-sized Petri  
203 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  
204 beans (as before). For females in the “male present” treatment group, a single newly emerged male of the  
205 same infection status and SeNP treatment was added to the female’s Petri dish. The beetles were allowed  
206 to reproduce for 72 h (with or without males present), during which time their reproductive output is at its  
207 greatest. A total of 96 females were set up in eight treatments (infection status  $\times$  SeNP application  $\times$  male  
208 presence), with 12 replicates for each treatment.

209

### 210 *Statistics*

211 The effects of infection status, SeNP concentration, and their interaction on the adult lifespan of male and  
212 female azuki bean beetles were tested with separate semiparametric Cox proportional hazards regression  
213 models. Because non-ovipositing females (virgin or not) have a longer life expectancy than ovipositing  
214 females (Yanagi and Miyatake 2003), females that did not produce viable eggs within the first 24 h were  
215 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  
216 L<sup>-1</sup>: -1; 100 mg L<sup>-1</sup>: -1; 500 mg L<sup>-1</sup>: -4).



217 The lifetime reproduction of females (total number of eggs) was evaluated with a generalized  
218 linear model (GLM) using a Poisson distribution and log link function, with infection status, SeNP  
219 concentration, and their interaction included as main effects. The daily reproduction of females (eggs per  
220 day) was assessed using a generalized linear mixed model (GLMM) with a Poisson distribution and log  
221 link function; infection status, SeNP concentration, and the number of days since emergence, as well as  
222 all interactions, were included as fixed effects, and the identity of individual females was included as a  
223 random effect. Because no viable eggs were produced more than 7 days after adult emergence, the  
224 analyses were limited to reproduction on days 0–7. As before, replicates that did not produce viable eggs  
225 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)  
226 (uninfected, 0 mg L<sup>-1</sup>: -1; 500 mg L<sup>-1</sup>: -1).

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

234 A multiple regression model was used to test the effects of infection status, SeNP treatment (0 mg  
235 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  
236 number of eggs laid per female over the 72-h period. Pairwise comparisons were made with FDR-  
237 corrected *p*-values. The same procedure was used to assess the effects of infection status, SeNP treatment,  
238 and the presence or absence of males (and all two-way interactions) on the logit-transformed proportion  
239 of hatched eggs produced per female over the 72-h period (see Warton and Hui 2011). Replicates that did  
240 not produce eggs (likely because the female did not mate prior to the study period) or in which the female  
241 (or male, if present) died within the 72-h period were excluded from the analysis (infected, 0 mg L<sup>-1</sup>,  
242 male absent: -2; male present: -1) (infected, 500 mg L<sup>-1</sup>, male absent: -4) (uninfected, 500 mg L<sup>-1</sup>, male  
243 absent: -2; male present: -1).

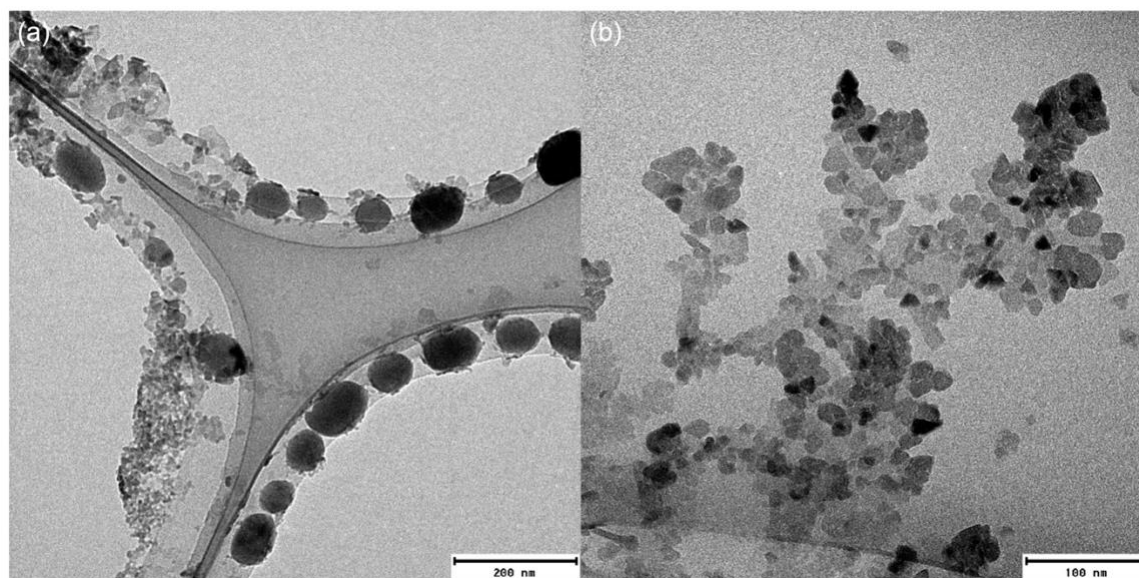
244 All statistical analyses were performed in R version 4.2.0 (The R Foundation for Statistical  
245 Computing 2022). The survivorship analysis also used the *survival*, *emmeans*, and *multcomp* packages;  
246 the fertility analysis used *lme4*, *optimx*, *afex*, *multcomp*, and *emmeans*; the behavioral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

249

## 250 **Results**

251 ***SeNP characterization***

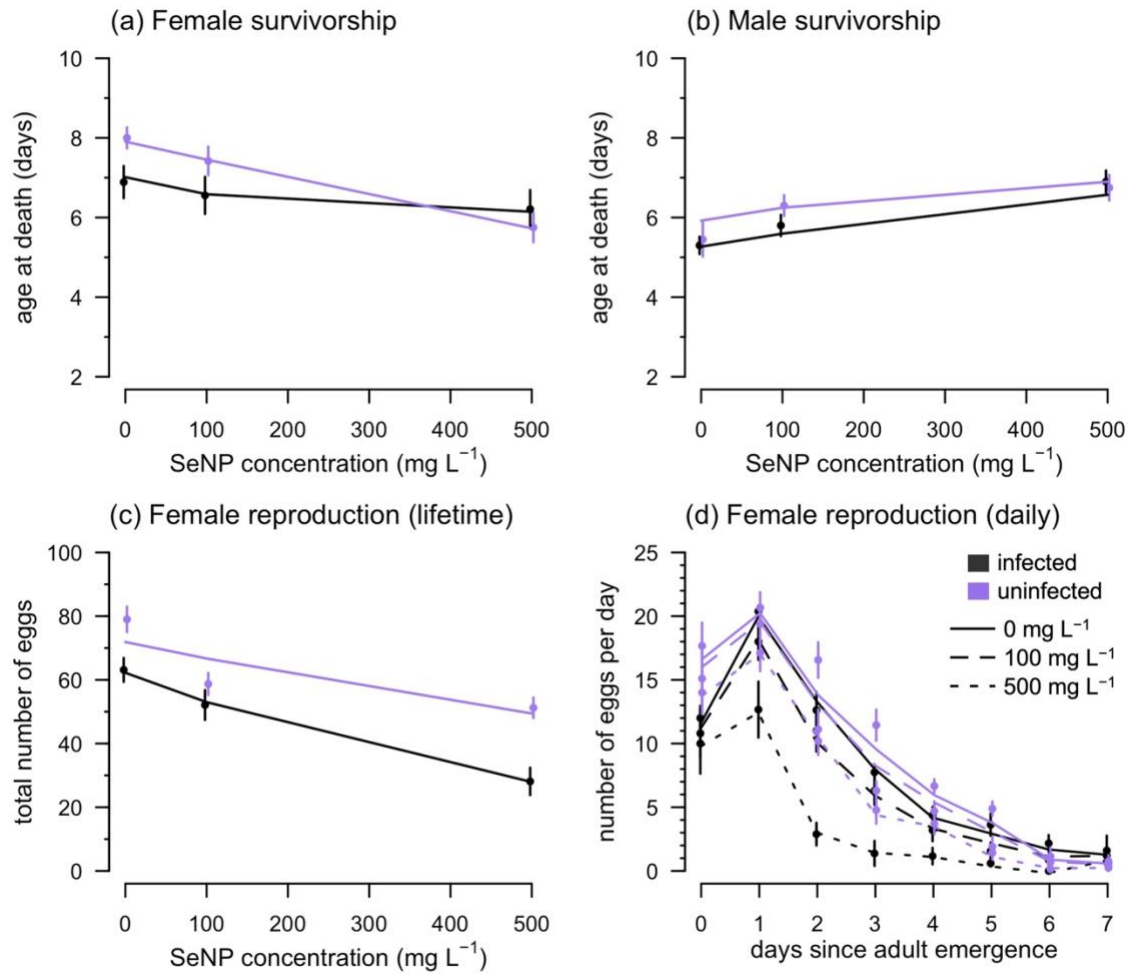
252 Reducing sodium selenite with ascorbic acid and stabilizing with polysorbate 20 yielded amorphous  
253 selenium nanoparticles (SeNPs) in two size classes (approximately 5–10 and 60–100 nm in diameter), the  
254 smaller of which were similar to the nanoparticulate debris produced by laser ablation and, likely during  
255 the desiccation processes for imaging, often self-polymerized into filamentous structures (Fig. 1).  
256



257  
258 **Fig. 1** Chemically synthesized selenium nanoparticles on a lacey carbon film microgrid. **a** Large and  
259 small amorphous SeNPs and **b** small, self-polymerizing SeNPs  
260

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

262 The adult lifespan of uninfected female azuki bean beetles was negatively affected by increasing SeNP  
263 concentration, whereas that of *Wolbachia*-infected females was unaffected by SeNP concentration  
264 (infection status  $\times$  SeNP concentration; Fig. 2a and Table 1). Also, while uninfected females tended to  
265 live longer than *Wolbachia*-infected females, this difference was not statistically significant ( $p = 0.084$ ;  
266 Fig. 2a and Table 1). The adult lifespan of male azuki bean beetles was greater for uninfected individuals  
267 than *Wolbachia*-infected individuals and was enhanced with increasing SeNP concentration (Fig. 2b and  
268 Table 1).  
269



270  
 271 **Fig. 2** Effects of SeNPs and *Wolbachia* infection on the lifespan and reproduction of adult azuki bean  
 272 beetles. **a** Female survivorship. **b** Male survivorship. **c** Female lifetime reproduction (total number of eggs  
 273 per female). **d** Daily reproduction (number of eggs laid per day). In all panels: points: means  $\pm$  SE; lines:  
 274 model predictions

275  
 276 **Table 1** Lifespan of female and male azuki bean beetles: analysis of deviance tables for the Cox  
 277 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

278

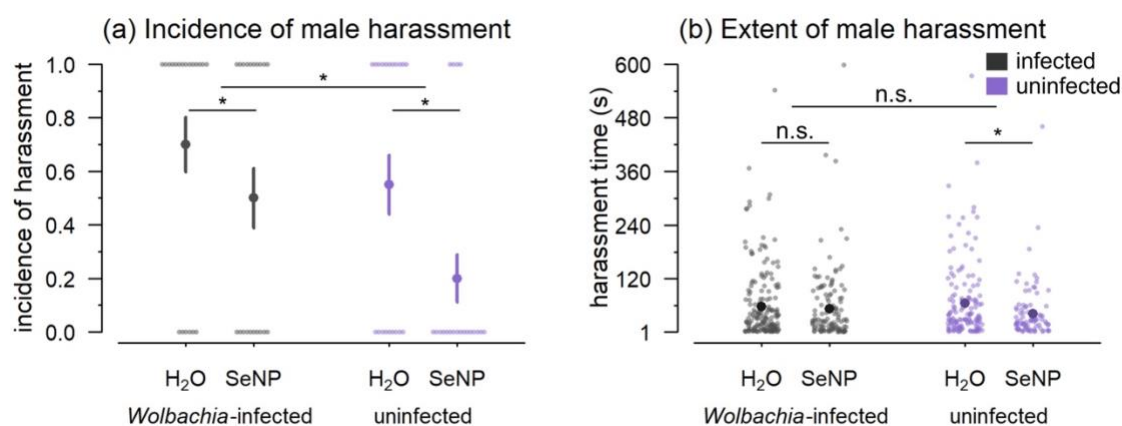
279 The lifetime reproduction of female azuki bean beetles was lower for *Wolbachia*-infected beetles  
 280 than for uninfected beetles and decreased with increasing SeNP concentration (Fig. 2c and Table 2).  
 281 Additionally, while SeNP concentration led to a sharper decline in the reproduction of *Wolbachia*-  
 282 infected females than for uninfected females, the rate of decline decreased as SeNP concentration  
 283 continued to increase (infection status  $\times$  SeNP concentration; Fig. 2c and Table 2). The daily reproduction  
 284 of females decreased with increasing SeNP concentration and over time (after peaking on day 1), and  
 285 while that of uninfected females tended to be greater than that of infected females, this trend was not  
 286 statistically significant (Fig. 2d and Table 2). Increasing SeNP concentration reduced the daily  
 287 reproduction of infected females more dramatically than uninfected females (infection status  $\times$  SeNP  
 288 concentration; Fig. 2d and Table 2). Although marginally significant ( $p = 0.052$ ), increasing SeNP  
 289 concentration tended to decrease daily reproduction from day 0, with the magnitude of this effect  
 290 changing over time; in general, the effects initially increased before decreasing with time (SeNP  
 291 concentration  $\times$  day; Fig. 2d and Table 2). On day 0, the effect of increasing SeNP concentration on the  
 292 daily reproduction of infected females was less pronounced than for uninfected females, and the  
 293 magnitude of the effect of increasing SeNP concentration increased for infected individuals on days 1 and  
 294 2, whereas the magnitude of the effect of increasing SeNP concentration remained relatively constant for  
 295 uninfected females, with a relatively small peak on day 3 (infection status  $\times$  SeNP concentration  $\times$  day;  
 296 Fig. 2d and Table 2).

297  
 298 **Table 2** Reproduction of female azuki bean beetles: analysis of deviance tables for the GLM of total  
 299 reproduction and the GLMM of daily reproduction

Predictor	Total reproduction			Daily reproduction		
	$\chi^2$	df	<i>p</i> -value	$\chi^2$	df	<i>p</i> -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>

301  
 302 **Male harassment and reproductive interference**  
 303 The incidence of male harassment behavior was lower in uninfected male–female pairs than in  
 304 *Wolbachia*-infected pairs, and was also lower when pairs were treated with 500 mg L<sup>-1</sup> SeNPs (Fig. 3a  
 305 and Table 3). The incidence of male harassment was not affected by an interaction between infection  
 306 status and SeNP treatment (Table 3). For male–female pairs in which harassment occurred, the duration

307 of male harassment behavior was unaffected by infection status, marginally decreased with SeNP  
 308 treatment, and significantly affected by an interaction between infection status and SeNP treatment—  
 309 SeNPs significantly decreased harassment behavior, but only in uninfected male–female pairs (Fig. 3b  
 310 and Table 4). The random effect (frailty) of male–female pair ID was also significant, indicating that the  
 311 typical duration of harassment behaviors varied from one pair to the next (Table 4).  
 312



313  
 314 **Fig. 3** Effects of infection status and SeNPs (0 or 500 mg L<sup>-1</sup>) on male harassment behavior in azuki bean  
 315 beetles. **a** Incidence of male harassment behavior during the 1-h observation period; points with solid bars  
 316 indicate the predicted values ± SE from the logistic regression model; asterisks indicate significant  
 317 differences between SeNP treatments and infection status. **b** Duration of male harassment over the 1-h  
 318 observation period; dark circles plot the mean predicted values ± SE from the Cox proportional hazards  
 319 model (SE are not visible because they are smaller than the circles); asterisks indicate significant  
 320 differences between SeNP treatments and between infection statuses  
 321

322 **Table 3** Incidence of male harassment behavior in azuki bean beetles: analysis of deviance table for the  
 323 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

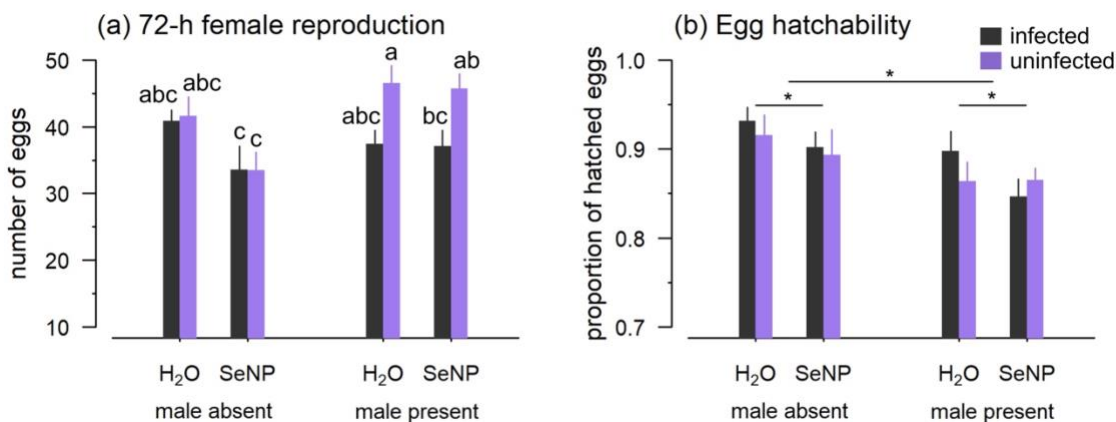
324

325 **Table 4** Duration of male harassment behavior in azuki bean beetles: analysis of deviance table for the  
 326 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	< <b>0.001</b>
infection status × SeNP trt.	94.16	19.67	< <b>0.001</b>

327  
 328 Female reproduction over a 72-h period was lower when individuals were infected with  
 329 *Wolbachia* or treated with SeNPs (Fig. 4a and Table 5); SeNPs also decreased egg hatchability (Fig. 4b  
 330 and Table 6). When males were present, female reproduction increased (Fig. 4a) while egg hatchability  
 331 decreased (Fig. 4b) (Table 6). There were also significant interactions between male presence and both  
 332 infection status and SeNP treatment on female reproduction, with male presence having a stronger  
 333 positive effect on the reproduction of uninfected females and SeNPs enhancing the positive effects of  
 334 male presence (Fig. 4a, Table 5); there were no other significant interactions for female reproduction or  
 335 hatchability. However, the *post hoc* analysis of 72-h reproduction was unable to resolve many differences  
 336 among the eight groups; water-treated uninfected females in the presence of males laid more eggs than  
 337 SeNP-treated infected females in the absence of males, and both water- and SeNP-treated uninfected  
 338 females in the presence of males laid more eggs than SeNP-treated infected or uninfected females in the  
 339 absence of males (Fig. 4a).

340



341

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

346

347 **Table 5** The 72-h reproduction of female azuki bean beetles: analysis of deviance table for the multiple  
 348 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 × SeNP trt.	0.03	1	0.864
infection status × male presence	5.58	1	<b>0.018</b>
SeNP trt. × male presence	3.85	1	<b>0.0497</b>

349  
 350 **Table 6** Proportion of hatched azuki bean beetle eggs: analysis of deviance table for the multiple  
 351 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 × SeNP trt.	1.03	1	0.309
infection status × male presence	0.31	1	0.577
SeNP trt. × male presence	0.03	1	0.856

352  
 353 **Discussion**

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

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

370 have reduced survivorship as selenium primarily accumulates in the Malpighian tubules (Hogan and  
371 Razniak 1991). In the house fly (*Musca domestica* L.; Diptera: Muscidae), the toxicity of selenium is  
372 reduced due to accumulation in the midgut, where cells sequester selenium in lysosomes (Simmons et al.  
373 1988). A related study of the rice meal moth (*Corcyra cephalonica* (Stainton); Lepidoptera: Pyralidae)  
374 found that selenium accumulates in the mitochondria (also noted by Simmons et al. 1988, and mostly in  
375 the mitochondrial membrane), which may actually have positive effects on mitochondrial energetics due  
376 to the presence of an unknown selenocysteine-containing protein or selenoenzyme (Lalitha et al. 1994).

377         Based on our hypothesis of *Wolbachia*-mediated resistance to SeNPs in the azuki bean beetle, our  
378 results partially supported the prediction that uninfected female beetles would be negatively affected by  
379 SeNPs via a reduction in adult lifespan and fecundity. Increasing concentrations of SeNPs decreased the  
380 lifespan of uninfected female beetles, whereas the lifespan of *Wolbachia*-infected females was not  
381 affected. The reproduction of infected and uninfected beetles generally decreased with increasing SeNP  
382 concentration, and these effects were less pronounced for *Wolbachia*-infected females than for uninfected  
383 females for the first 24 h post-exposure; however, over time, SeNPs reduced the total reproduction of  
384 infected females more than that of uninfected females. Because of the close association of *Wolbachia*  
385 with host reproductive tissues (Ijichi et al. 2002), this suggests the existence of a set of life history trade-  
386 offs with respect to endosymbiont-mediated resistance to SeNPs. On the contrary, in males, increasing  
387 concentrations of SeNPs enhanced lifespan regardless of infection status, although this does not  
388 necessarily contradict our prediction—we did not expect SeNPs to have the same effects for beetles of  
389 both sexes due to fundamental differences in physiology and the dynamics of infection (Yanagi and  
390 Miyatake 2003, Wagner and Bakare 2016, Ijichi et al. 2002). In the small brown planthopper (*Laodelphax*  
391 *striatellus*), *Wolbachia* may not only enhance the immune system by altering host gene expression, but, in  
392 males, also increases the expression of genes related to the metabolism of selenocompounds (Liu et al.  
393 2019). Interestingly, in the Gulf Coast tick (*Amblyomma maculatum* Koch; Arachnida: Ixodida:  
394 Ixodidae), the selenoprotein thioredoxin reductase (TrxR) plays an important role in structuring the  
395 bacterial community in the microbiome (Budachetri and Karim 2015). Future studies should explore the  
396 physiological mechanisms by which *Wolbachia* mediates the response to SeNPs in seed beetles.

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



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

414 In a related species, the lifetime reproductive effort in males is equal to that of females (Wagner  
415 and Bakare 2016), so a reduction in male sexual behaviors may redirect significantly more resources  
416 towards male survival. Yet this overall decrease in behavioral activity raises the question of whether  
417 SeNPs truly have a stimulatory effect on male *C. chinensis*, so it may not be entirely accurate to describe  
418 this response as “hormesis” (it is also currently unknown at what dose SeNPs would be lethal to males,  
419 although, in general, the toxicity of NPs is not comparable to that of conventional insecticides—SeNPs  
420 might be better described as “entomotoxic” as opposed to “insecticidal” *sensu stricto*; e.g. Debnath et al.  
421 2011). It may also be that SeNPs help reduce sexual conflict by calming males after being rejected by a  
422 partner (thus reducing the incidence or recurrence of harassment behavior). Regardless, the presence of  
423 males tended to increase female reproduction (especially when treated with SeNPs), which may indicate a  
424 shift in the risk-benefit dynamics associated with a lower frequency of remating in nutrient-poor  
425 conditions (such as a stored product setting): male harassment and injuries sustained during copulation are  
426 minimized while nuptial gifts (water or nutrients) are still available (Miyatake and Matsumura 2004,  
427 Harano et al. 2006, Rönn et al. 2006, Sakurai and Kasuya 2008, Harano 2015; for an example with singly  
428 mated *C. chinensis*, see Yanagi and Miyatake 2003; for examples involving *C. maculatus*, see Fox 1993,  
429 Arnqvist et al. 2005, Gay et al. 2009, den Hollander and Gwynne 2009).

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

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

440

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

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

461

#### 462 **Conclusion**

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

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

474

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479

## 480 **Declarations**

### 481 *Ethical approval*

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

483

### 484 *Competing interests*

485 The authors declare no conflicts of interest or competing interests.

486

### 487 *Authors' contributions*

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

492

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496

### 497 *Availability of data and materials*

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

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