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RUNNING HEAD: Harrison and Preisser: Factors affecting aphid antipredator responses

TITLE: Dropping behavior in the pea aphid: how does environmental context affect anti-predator responses? Katharine V. Harrison¹ and Evan L. Preisser^{1*}

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ABSTRACT

The pea aphid *Acyrtosiphon pisum* Harris (Hemiptera: Aphididae) is a phloem-feeding insect whose anti-predator defenses include kicking, walking away, and dropping from the plant. Aphid dropping, a risky and energetically-costly antipredator behavior, can be increased by the release of aphid alarm pheromone; there is also evidence that insect density and plant health can affect the likelihood of aphids engaging in this behavior. We investigated whether interactions between alarm cues, insect density, and plant health can alter the dropping behavior of aphids in response to an artificial disturbance. The presence of the alarm pheromone E-β-farnesene resulted in a nearly 15-fold increase in aphid dropping behavior; the other two factors, however, did not affect dropping and none of the two- or three-way interactions were significant. This was surprising, since aphids affected plant health: production of new plant biomass after five days of exposure to high aphid densities was 50% lower than in the control treatment. This research adds to our understanding of the factors affecting aphid anti-predator behavior; the fact that neither aphid density nor feeding period impacted dropping may reflect the high energetic costs of this activity and an unwillingness to use it in any but the riskiest situations.

KEYWORDS

Pea aphid, *Acyrtosiphon pisum*, non-consumptive effect, anti-predator behavior, alarm pheromone, predator, prey, dropping, E-β-farnesene, density

38 **INTRODUCTION**

39 Predators affect prey directly, via consumption (i.e., consumptive effects), and indirectly,
40 via the induction of energetically costly anti-predator responses (i.e., non-consumptive effects).
41 These responses can include changes in prey behavioral, morphological, or developmental traits
42 (Petranka et al. 1987, Lima 1998, Podjasek et al. 2005, Preisser et al. 2005, Verheggen et al.
43 2009). While these pathways differ in their benefit to the predator, both can negatively affect the
44 prey: behaviors that lessen predation risk can also increase energy expenditure, reduce feeding
45 time, and/or expose prey to alternative predators (Losey and Denno 1998a, Nelson 2007).
46 Because only a small fraction of prey individuals are eaten but a much larger fraction respond to
47 predation risk, the cumulative impact of non-consumptive effects on prey population growth may
48 equal or exceed that of direct consumption (Nelson et al. 2004, Preisser et al. 2005).

49 Because predators and prey interact in a dynamic environment, the magnitude of predator
50 indirect effects on prey can also vary. Many mobile prey use behavioral tradeoffs to balance the
51 rewards of foraging and mating opportunities with the risk of consumption, and may respond to
52 several different types of predator cues (Lima 1998). Larvae of two-lined salamanders (*Eurceya*
53 *bislineata*) and Cope's grey treefrog (*Hyla chrysoscelis*), for instance, both avoid water
54 previously occupied by their predator, the green sunfish (*Lepomis cyanellus*); exposure to fish
55 cues also causes *H. chrysoscelis* tadpoles to spend more time in refuges (Petranka et al. 1987).
56 Refuges are also important to invertebrate prey: larvae of the thrip *Frankliniella occidentalis*
57 take refuge in spider mite webs to avoid the predatory mite *Neoseiulus cucumeris* (Magalhaes et
58 al. 2007). Hunger also alters prey responses to risk: while remaining hidden avoids predators,
59 prey must forage to survive. Starved mayfly (*Baetis caudatus*) larvae, for example, expose

60 themselves to higher predation risk than less hungry individuals by spending more time in risky
61 but resource-rich food patches (Kohler 1989).

62 The family Aphididae includes many phloem-feeding species that occur in natural and
63 managed systems and are consumed by an array of invertebrate predators. One common member
64 of this family, the pea aphid *Acyrtosiphon pisum* (Harris), is an agricultural pest that can
65 quickly reach high densities because of its ability to reproduce parthogenetically during the
66 summer growing season. While *A. pisum* move slowly and possess few morphological defenses,
67 they have developed anti-predator behaviors to evade or deter predators. These behaviors include
68 kicking, stylet removal, walking away, and dropping from the plant (Roitberg 1978).

69 Dropping behavior in *A. pisum* can be influenced by several factors, including the risk of
70 predation, value of the plant resource, and mortality risk in the new environment (Losey and
71 Denno 1998b). This behavior reduces feeding time and reproduction, increases the risk of
72 desiccation, and exposes aphids to ground predators (Losey and Denno 1998a, Nelson 2007).
73 The substantial costs of aphid dropping suggest that the frequency of this antipredator behavior
74 should change when aphids are reared on host plants whose nutritive value has been reduced by
75 prior herbivory (Denno et al. 1986, Awmack and Leather 2002). The generally negative
76 relationship between plant health and aphid density, and the decrease in aphid performance
77 (indicated by both aphid growth and the increased production of winged individuals) as plant
78 health declines, has been extensively documented in the scientific literature (e.g., Müller et al.
79 2001). When food-deprived *A. pisum* are exposed to a parasitoid, for example, they use less
80 energetically-costly behaviors (kicking) than do well-fed aphids that drop from the plant or walk
81 away from the threat (Villagra et al. 2002). This response is not restricted to *A. pisum*; knapweed
82 aphids, *Uroleucon jaceae*, also reduce their dropping rate when feeding on low-quality plants

83 (Stadler et al. 1994). Conversely, dropping rates might decrease on high-quality plants if the
84 benefit of staying on such a host exceeds the cost of reduced dropping (Stadler et al. 2002). One
85 study, for instance, found that *A. pisum* on high-quality diet dropped less often in response to risk
86 cues than those reared on low-quality diet (Dill et al. 1990).

87 Threatened aphids also secrete an alarm pheromone, consisting of E- β -farnesene and
88 other sesquiterpene hydrocarbons such as α - and β -pinene (Pickett and Griffiths 1980), that
89 elicits an array of anti-predator behaviors in other aphids (Roitberg 1978). Response to this
90 pheromone can be affected by the aphids' environment. Pea aphids exposed to E- β -farnesene
91 were more likely to drop with increasing amounts of alarm pheromone and when the pheromone
92 cue is preceded by a vibrational cue indicative of a predator (Clegg and Barlow 1982). Pea
93 aphids are particularly sensitive to this alarm cue; a larger portion of *A. pisum* drop in response to
94 E- β -farnesene than any other aphid species sensitive to this alarm pheromone (Montgomery and
95 Nault 1977). Antipredator behavior in some aphid species is also sensitive to population density.
96 In the wheat aphid, *Schizaphis graminum*, dropping behavior in response to a coccinellid
97 predator increases as a function of population density; while similar work on *A. pisum* did not
98 find a relationship between aphid density and dropping, densities lower than 30 aphids per plant
99 were not tested (McConnell and Kring 1990, Losey and Denno 1998b).

100 We report the results of research assessing how interactions between prey density, alarm
101 cues, and food resources affect aphid responses to predation risk. Specifically, we tested how
102 dropping behavior in response to an artificial predator differed at low versus high aphid
103 densities, in the presence or absence of alarm cues, and on host plants that had been fed upon for
104 a short or long time period. Our findings add to the results of previous research by addressing
105 how the potential interplay between these factors can affect aphid antipredator defense.

106 **MATERIALS AND METHODS**

107 Pea aphids were collected from pea plants (*Pisum sativum*) cultivated at the agronomy
108 farm at the University of Rhode Island, Kingston, RI, USA in September of 2014. Aphids were
109 reared in the laboratory on broad bean plants (*Vicia faba* L. var. “Varoma” source: Johnny’s
110 Selected Seeds, Maine, USA). Seeds were planted in 26 grams of potting mix (Metro-mix 360;
111 Sun Gro Horticulture, Agawam, MA, USA). Bean plants emerged approximately one week after
112 planting. Aphids were maintained in mesh-sided insect cages containing two two-week-old *V.*
113 *faba* plants. Two fresh plants were added to each insect cage every five days, and aphids were
114 given twelve hours to transfer to the new plants before the old plants were removed and
115 composted. Plants and insects were raised in a temperature-controlled laboratory at 22°C and
116 16L:8D photoperiod under fluorescent shop lights.

117 Prior to the start of an experimental trial, eight uninfested two-week-old *V. faba* plants
118 were trimmed to two leaves per plant and individually potted in a 20 cm plastic pot. After 24
119 hours, each pot-plant combination was covered with a cylindrical lid made of clear plastic
120 transparency film and dacron chiffon netting (70 per inch mesh size, 240-micron hole size). The
121 netting allowed light and air to pass through while remaining impermeable to aphids. Each pot,
122 plant and lid combination was used as an individual experimental arena.

123 ***Experimental design***

124 We tested how *A. pisum* dropping behavior was affected by aphid density (low, high),
125 length of feeding period (short, long), and exposure to aphid alarm cues (present, absent). We
126 tested aphid density by transferring five (low density) or 50 (high density) aphids onto individual
127 *V. faba* plants. These densities reflect those found during a field survey on the least- and most-
128 infested quartiles of leaves on aphid-colonized *V. faba* plants (Preisser, *unpublished data*). We

129 tested the length of feeding period by allowing aphids to feed on a previously-uninfested plant
130 for one (short feeding period) or five days (long feeding period). We tested exposure to alarm
131 cues by exposing aphids to a crushed aphid (E- β -farnesene present) or neutral cue (same
132 disturbance regime without the cue). We assessed the positive and control cue in each of the four
133 main experimental groups listed above for a total of eight experimental groups. All three factors
134 were crossed for a total of eight ($2*2*2$) experimental treatments, and each treatment was
135 replicated eight times (=64 total replicates).

136 We started each replicate by using a damp fine-point paintbrush to gently transfer an
137 appropriate number of aphid nymphs from the source population onto an experimental plant.
138 Although immature aphids are more conservative in their dropping behavior than adult aphids
139 (Roitberg and Myers 1978, Gish et al. 2012), we chose to use third-instar nymphs in order to
140 maintain the initial aphid densities (i.e., prevent the aphid population from growing) throughout
141 the experiment. Immediately following aphid transfer, we re-counted aphids on each
142 experimental plant to ensure that none had fallen off during the transfer process. Replicates from
143 the eight experimental treatments were randomly interspersed on a metal shelf underneath
144 fluorescent shop lights (light and temperature conditions as above) to prevent spatial bias. In
145 order to measure the impact of aphid feeding on plant growth, we also included five control
146 plants that were treated similarly (i.e., removal of all but two leaves, mimicking of disturbance
147 caused by aphid inoculation) but did not have aphids added to them.

148 We added first-instar nymphs in the five-day treatments in order to prevent aphid
149 reproduction; third-instar nymphs were used for the one-day treatments. Because most of the
150 first-instar nymphs matured into third-instar nymphs by the end of the five-day treatment, our

151 tests of dropping behavior primarily assessed third-instar nymphs in both the short- and long-
152 feeding-period treatments.

153 Aphid dropping behavior in a given replicate was tested either one or five days after the
154 experiment began. Our testing protocol consisted of exposing aphids for two minutes to a
155 paintbrush with or without a crushed aphid cue. During the two minutes, the paintbrush was
156 firmly brushed onto parts of the leaf immediately adjacent to aphids for 10-15 seconds to
157 simulate predator presence, but did not physically contact any aphids. At the end of the two-
158 minute testing period, both dropped and remaining aphids were counted. The above-ground
159 biomass of plants in the five-day and control treatment was then divided up into old growth and
160 new growth (i.e., biomass added between the start and end of the five-day period). The old- and
161 new-growth biomass of each plant was then weighed in order to determine the percentage of
162 plant growth after five days in the control (zero aphids/ plant), low density (five aphids/ plant),
163 and high density (50 aphids/ plant) treatments.

164 *Statistical Analysis*

165 We tested whether our aphid densities affected plant resources using a one-way ANOVA
166 to compare the amount of new growth per plant after five days in the control, low density and
167 high density treatments. We tested the impact of risk cues, aphid density, and feeding period on
168 the arcsine square-root transformed proportion of aphids dropping using a fully-factorial three-
169 way ANOVA. Data was checked prior to analysis to ensure that it was normally distributed and
170 that variances were homogeneous. While the data from the cue present treatments met both
171 criteria, the data from the cue absent treatments did not (because so few aphids dropped);
172 ANOVA is, however, highly robust to departures from both assumptions when per-treatment
173 sample sizes are large (Underwood 1997). JMP 9.0.0 (SAS 2010) was used for all analyses.

174 **RESULTS**

175 Aphid feeding over a five-day period did not impact the weight of existing plant tissue
176 (F_{2,21} = 0.06, p = 0.95), but substantially reduced the amount of new plant growth (F_{2,21} = 6.48, p
177 = 0.006). As a result, new growth made up 13.4% and 27.6% of total aboveground biomass in
178 the 50-aphid and aphid-free treatments, respectively (Tukey's HSD with p = 0.05, Fig. 1).

179 Aphid alarm cues increased the percentage of aphids dropping, from 1.5% in cue-absent
180 treatments to 22.4% in cue-present treatments (F_{1,40} = 44.9, p < 0.01; Fig. 2). Despite the
181 decrease in new plant growth associated with aphid feeding (Fig. 1), neither feeding period nor
182 aphid density affected dropping behavior (F_{1,40} = 1.27 and 0.21 respectively, both p > 0.20).
183 There were also no significant two- or three-way interactions between any of the main effects.

184 **DISCUSSION**

185 The presence of aphid alarm cues (in this case, a crushed prereproductive aphid)
186 increased dropping rates 15-fold, with 22.4% of aphids dropping in the cue-present treatments
187 versus 1.5% in the cue-absent treatments. E-β-farnesene, the substance previously identified as
188 the predominant component of aphid alarm pheromone (Pickett and Griffiths 1980), is an active
189 volatile terpenoid in aphid cornicle droplets; the results of our experiment are consistent with
190 previous research finding that even prereproductive aphids reared in the absence of predators
191 produce E-β-farnesene (Bowers et al. 1972, Mondor et al. 2000, Francis et al. 2005). Previous
192 work has shown that the volatiles emitted from crushed aphids are nearly identical to those
193 produced by cornicle droplets, and that aphids show similar defensive responses to both
194 substances (Pickett and Griffiths 1980).

195 While we anticipated that aphid alarm cues increased dropping rates, we were surprised
196 at how few aphids dropped in their absence. In all of the treatments, we used a fine-point

197 paintbrush to disturb the leaves and approach (although not touch) each individual aphid.
198 Although the aphids would sometimes respond to the paintbrush by moving away or kicking,
199 they apparently did not view the combined visual and vibrational stimulus presented by the
200 paintbrush as an immediate threat (as per Ben-Ari and Inbar 2014). While vibration in
201 combination with E- β -farnesene can increase dropping behavior in younger instars, the act of
202 dropping is costly and can reduce fecundity or increase mortality (Roitberg 1978, Nelson et al.
203 2004, Nelson 2007). Additionally, cornicle droplets are high in triglycerides and are costly for
204 aphids to synthesize; the secretion of a single cornicle drop can delay offspring production
205 (Callow et al. 1973, Mondor and Roitberg 2003). The high cost of E- β -farnesene should limit its
206 use to the riskiest of situations and make it a highly reliable indication of imminent danger
207 (Orrock et al. 2015). In the absence of an alarm pheromone, however, the vibrational and visual
208 cues presented by the paintbrush alone were insufficient to induce dropping in our experiment.

209 Although increased aphid densities reduced plant growth (Fig. 1), neither aphid density
210 nor feeding period affected aphid dropping behavior (Fig. 2). In addition, neither factor
211 interacted with the presence/absence of aphid alarm cues. The lack of a density effect may be
212 explained by the fact that our numbers were too low to affect aphid behavior. While previous
213 work on the greenbug *Schizaphis graminum* found that dislodgement increased with increasing
214 aphid density, aphid numbers in their low-density treatment were similar to those of our high-
215 density treatment (McConnell and Kring 1990). In contrast, Losey and Denno (1998b) used
216 densities similar to ours and found no relationship between pea aphid density and dropping
217 response. Aphid numbers in our high-density treatment were chosen to increase the likelihood
218 that their host plants, which we trimmed to two leaves to aid in aphid detection, would survive
219 five days of aphid feeding. While plant growth in the high-density treatment was significantly

220 lower than in the no-aphid treatment, the impact of this reduction in growth on plant quality
221 appeared insufficient to alter aphid dropping behavior. It is possible, however, that less
222 energetically-costly aphid defensive behaviors such as kicking or walking away may be more
223 sensitive to changes in density than aphid dropping (Villagra et al. 2002). Since immature aphids
224 are less likely than adults to drop in response to risk (Roitberg and Myers 1978, Gish et al.
225 2012), it is also worth noting that adult aphids may exhibit different responses.

226 Our results suggest a number of ways to modify our experimental design in order to
227 better study *A. pisum* dropping behavior as a function of density and plant health. First,
228 increasing aphid numbers in the high-density treatment above 50 insects/plant should magnify
229 the insects' effect on plant health and increase our ability to detect differences between the low-
230 and high-density treatments. Second, our one- versus five-day feeding period treatments, which
231 were intended to assess the importance of plant quality, could be replaced with treatments in
232 which plants were either protected from or exposed to feeding prior to their experimental use as
233 seen in Tokunaga and Suzuki (2007). The length of aphid feeding period could also be extended
234 in order to include the next generation of aphids and observe any transgenerational effects of E-
235 β -farnesene exposure, aphid density, and/or plant health. The alarm pheromone E- β -farnesene
236 has been shown to modulate transgenerational wing induction in *A. pisum* and solitary aphids
237 produce less E- β -farnesene than do aphids with conspecifics (Podjasek et al. 2005, Verheggen et
238 al. 2009). Nymphs of maternal aphids exposed to alarm pheromone also select less risky feeding
239 sites, a decision which alters colony structure (Keiser and Mondor 2013). As a result, it is
240 possible that the impacts of our treatments can only be observed across a longer time period than
241 the one we chose.

242 Although we did not see an impact of aphid density or plant health on dropping behavior,
243 our results still contribute to our understanding of aphid anti-predator responses, and the
244 conditions in which they do (or do not) manifest. The fact that aphids responded strongly to
245 alarm pheromone demonstrated that they can alter their dropping behavior; the fact that the other
246 factors did not induce similar variation may reflect the energetic and fitness cost of this behavior.
247 By demonstrating how reluctant aphids can be to increase dropping in response to all but the
248 most serious threat (i.e., alarm pheromone), our findings thus add to the results of previous
249 research addressing into the factors affecting aphid antipredator defense.

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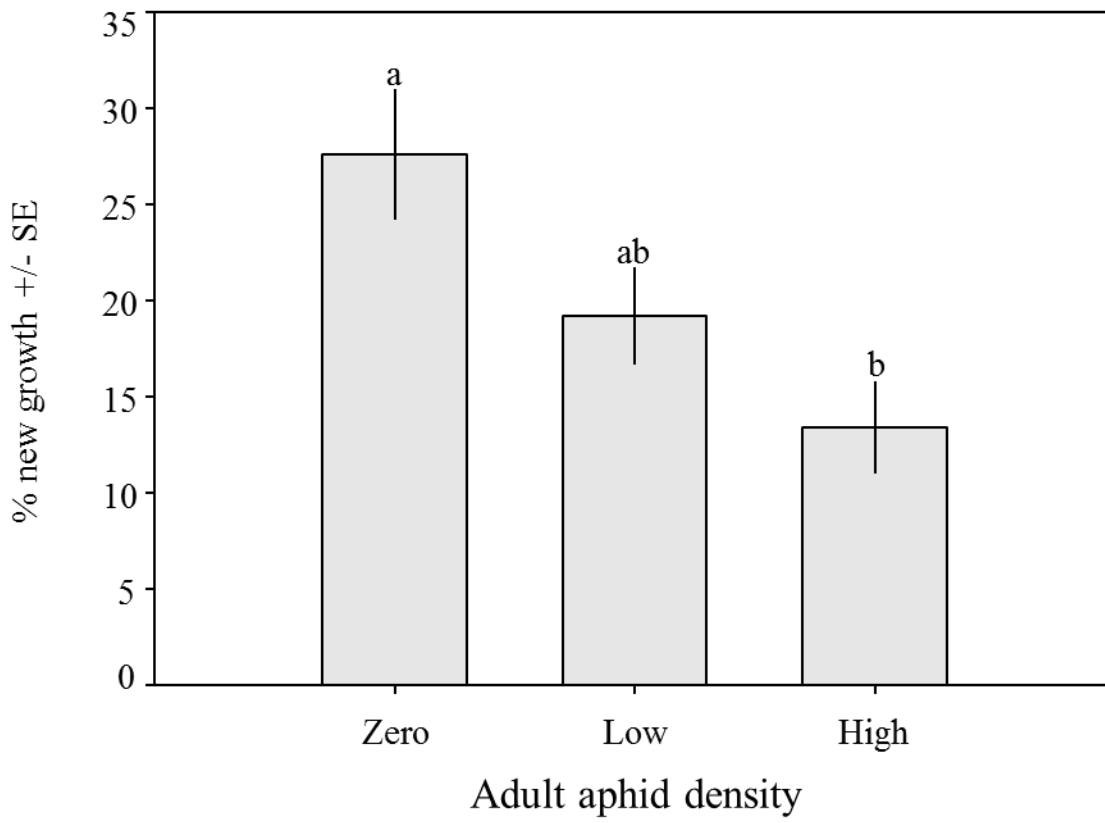
335 **Figure Legends**

336 Figure 1. Effect of aphid density on percent new plant growth (mean \pm standard error)
337 over a five-day feeding period. The percentage of aboveground plant biomass made up of new
338 growth was significantly lower in the high-aphid density treatment than in the zero-aphid control
339 (13% and 27%, respectively; $F_{2,21} = 6.48$, $p = 0.006$). Bars with different letters represent
340 significant differences (Tukey's HSD, $\alpha = 0.05$).

341 Figure 2. Effect of alarm cue, aphid density, and feeding period on aphid dropping (mean
342 \pm standard error) in response to disturbance. The presence of an alarm cue significantly increased
343 the percentage of aphids dropping across all treatments ($F_{1,40} = 36.5$, $p < 0.01$). There was no
344 significant effect of aphid density or length of feeding period (both $p > 0.05$), and there were no
345 significant interactions between any of the main effects. Bars with different letters represent
346 significant differences (Tukey's HSD, $\alpha = 0.05$)

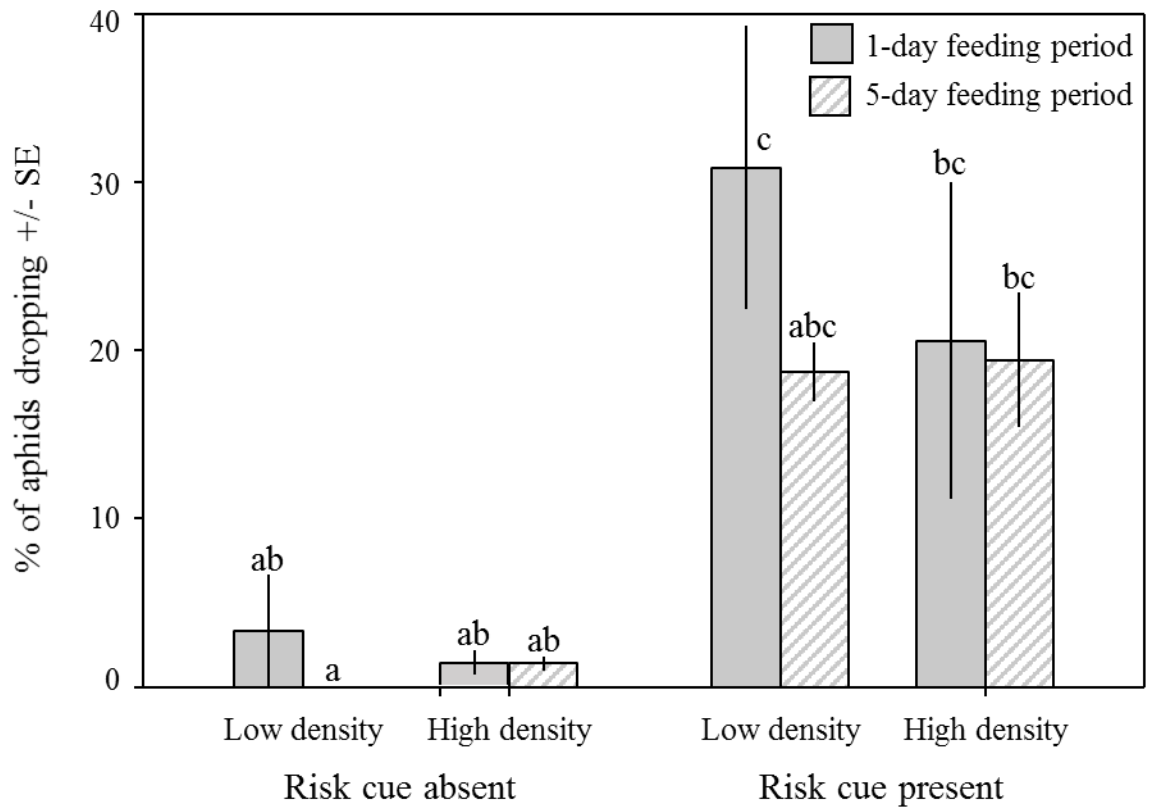
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Figure 1.



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Figure 2.



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