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Do Bioflavonoids in *Juniperus virginiana* Heartwood Stimulate Oviposition in the Ladybird *Coleomegilla maculata*?

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ABSTRACT: Maximizing the reproductive potential of ladybird beetles fed factitious foods or artificial diets, in lieu of natural prey, is a major challenge to cost-effective mass rearing for augmentative biological control. In this study, we tested the hypothesis that compounds in redcedar, *Juniperus virginiana*, stimulate oviposition in the ladybird *Coleomegilla maculata*. We also tested the prediction that several bioflavonoids, identified in heartwood fractions, elicited this behavioral response. Phenolic compounds were extracted from *J. virginiana* heartwood sawdust, separated into several fractions, then presented to adult beetles, in a powdered, pure form, in the laboratory. Females preferentially oviposited within 1 to 2 cm of fractions B, C, D, and E, but not A or the unfractionated extract, at the base of test cages. Chemical analysis identified bioflavonoids in heartwood fractions and subsequent bioassays using several identified in fractions C, D, and E confirmed that quercetin, taxifolin, and naringenin (to a lesser extent) stimulated oviposition. All tested fractions and bioflavonoids readily adhered to the chorion of freshly laid eggs but did not reduce egg hatch. This study demonstrates that several bioflavonoids stimulate oviposition by *C. maculata* and could be useful for mass rearing programs.

KEYWORDS: Chemical ecology, Coccinellidae, Cupressaceae, oviposition, pink-spotted lady beetle, predator

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Introduction

Coleomegilla maculata De Geer (Coleoptera: Coccinellidae) is a native ladybird beetle found in natural and managed ecosystems in North, Central, and South America.[1–4] It is polyphagous, consuming eggs and young larval stages of many small, soft-bodied insects and mites, with a proclivity for aphids[5–7] and plant pollen.[8,9] There is interest in using this ladybird beetle for applied biological control of insects and mites in protected plant systems, ie, nurseries, high tunnels, gardens, greenhouses, and plantscapes.[10,11] Mass production of *C. maculata* over multiple generations will be a requisite to generating the quantity of individuals necessary for augmentative releases into protected plant systems. Our research aims to discover efficient, low-cost methods to mass produce *C. maculata* and other ladybirds.

One of the challenges to mass produce ladybirds and other predators at low cost is the identification of inexpensive alternative foods, ie, artificial diets or factitious prey.[12–14] Unfortunately, alternative foods often lack specific compounds that stimulate feeding; these stimulants are often found in natural prey. Oftentimes, oviposition is stimulated by the availability of natural (essential) prey, in abundance and of high quality.[15–18] Thus, a major challenge is stimulating *C. maculata* and other ladybirds to oviposit their full potential of eggs when restricted to feeding on alternative foods rather than natural prey (eg, aphids). In addition, cues that guide ovipositing females to suitable sites for egg laying are often connected to natural prey. In the field, many ladybirds, including *C. maculata*, prefer ovipositing on leaves and other substrates in the vicinity of prey, but not always.[19–21] In artificial rearing systems, devoid of natural prey of high quality, females may or may not rely on other cues (either plant or insect derived) to choose an oviposition site.[22,23] Females may choose to lay eggs on artificial oviposition substrates such as tissue paper, filter paper, corrugated cardboard, aluminum sheets, and other materials,[24–28] in lieu of plant leaves. In the absence of any oviposition substrate, females might lay their eggs on the sidewalls or the underside of the lid of the rearing vial, container, or Petri dish.[18,29]

Very few synthetic compounds are known to stimulate and enhance oviposition in ladybirds. The fatty acids, myristic acid and stearic acid, when formulated within an artificial diet, enhanced oviposition in the ash-gray lady beetle *Olla v-nigrum* (Mulsant).[30] When formulated with an alternative food, ie, powdered brine shrimp eggs, *Artemia franciscana* Kellogg (Anostraca: Artemiidae) plus 5% palmitic acid stimulated oogenesis (egg maturation) in *C. maculata*.[31]

Other plant-derived natural products might be capable of stimulating oviposition in ladybirds. A water-based extract from

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the leaves and wood of *Berberis vulgaris* L. (European barberry) stimulated oviposition in 2 ladybirds, *Adalia bipunctata* (L.) and *Coccinella septempunctata* L., when it was sprayed on the leaves of *Prunus cerasus* L. (sour cherry), which was not a preferred oviposition site, prior to treatment.[32] Eastern redcedar, *Juniperus virginiana* L. (Pinales: Cupressaceae), wood panels, and wood extracts, including several fractions, have been shown to stimulate oviposition in 4 ladybirds: *A. bipunctata, Coccinella transversoguttata* Faldermann, *Cycloneda munda* (Say), and *C. maculata*.[33,34] Polyphenols in *J. virginiana* wood were thought to be responsible for the stimulatory responses in these ladybirds when they came in contact with fractions in laboratory bioassays.[33,34] Polyphenols are found in a diversity of plants and some are known to defend plants from attack by pathogens, parasites, and herbivorous insects.[35,36] A class of polyphenols, ie, bioflavonoids, are responsible for the color of flower petals, attract pollinating insects to flowers (and pollen) and herbivorous insects to their host plants.[37–41] Many ladybirds, including *C. maculata*, are attracted to plant pollen and could be using bioflavonoids, or oxidative by-products from bioflavonoids, to orient to oviposition sites near prey (eg, aphids) in the field.

In this study, we tested the hypothesis that compounds in *J. virginiana* heartwood stimulate oviposition in *C. maculata*. We also tested the prediction that several bioflavonoids, identified in heartwood fractions, elicited this behavioral response. The objectives of this study were to (1) evaluate the effects of *J. virginiana* fractions on *C. maculata* oviposition, (2) identify specific polyphenols (ie, bioflavonoids) in fractions, and (3) evaluate the effects of several synthetic bioflavonoids on *C. maculata* oviposition.

Materials and Methods

Insect colonies and food sources

Our *C. maculata* colony originated from adults provided by US Department of Agriculture (USDA) colleagues in Beltsville, MD and Brookings, SD, USA. Life stages (eggs, larvae, pupae, and adults) were reared separately in plastic containers in an environmental room (24°C, 50%-60% relative humidity [RH], 16 hours photophase, year-round). Immature stages were kept in medium-sized Petri dishes (2.5-cm high, 9.0-cm diameter), whereas adult mating pairs were held in plastic cages (10-cm high, 8-cm diameter), with screened lids, and crumbled facial tissue paper (Kleenex; Kimberly-Clark Corp., Neenah, WI, USA) was used as an oviposition substrate in cages. Egg clutches were routinely harvested from tissue paper, as needed, for experiments or for the furtherance of the colony. At the time that we commenced this study, we had maintained this colony for more than 25 consecutive generations without introducing any "wild-type" individuals from the field. Adults and larvae were fed an excess of frozen-fresh moth eggs *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) or freeze-dried brine shrimp eggs *A. franciscana*, twice a week. A cotton wad, moistened with distilled water, was provided in rearing cages at all times.

Sample preparation and extraction

Analytical sample preparation and extraction. A *J. virginiana* tree from Tazewell County, IL, was harvested and heartwood sawdust was prepared as described in detail in a previous study.[42] To summarize, heartwood sawdust was extracted using supercritical fluid extraction with carbon dioxide to remove the essential oils, then with pressurized ethanol to obtain the extract containing the phenolics.

Preparative chromatographic purification. For phenolic purification, approximately 25 g of extract powder was resuspended in methanol for 24 hours. The resulting methanol extracts were concentrated by rotoevaporation and resuspended in water. A Büchi (Newcastle, DE, USA) Sepacore flash chromatography system with dual C-605 pump modules, C-615 pump manager, C-660 fraction collector, C-635 UV photometer, with Sepacore Record chromatography software, was used, a 40 mm × 150 mm flash column with approximately 90 g of preparative C18 reversed-phase bulk packing material (125 Å, 55-105 µm; Waters Corporation, Milford, MA, USA). The column was equilibrated with 20% methanol and 0.5% acetic acid in water for 5 minutes at a flow rate of 30 mL/min. After injection of the samples (20 mL), the column was developed with a binary gradient to 100% methanol over 50 minutes. The effluent was monitored at 280 nm and fractions based on absorbance were collected in the fraction collector by the software program. Six broad overlapping peaks and a wash fraction were eluted from the column (fractions A-G). The procedure was repeated until all the extract was used. Fractions containing each absorbance peak were pooled and concentrated by evaporation in the hood at room temperature.

For further purification, selected fractions were run on a Shimadzu (Columbia, MD, USA) preparative high-performance liquid chromatography (HPLC) system with dual 8A pumps, SIL 10vp autoinjector, SPD M10Avp photodiode array (PDA) detector, FRC-10A fraction collector, and SCL 10Avp system controller, all operating under the Shimadzu Class VP operating system. Sample aliquots of 10 mL in methanol were injected on a Phenomenex (Torrance, CA, USA) Luna C18 semipreparative reversed-phase column (10 µ, 100 Å, 250 cm × 50 cm). The column was pre-equilibrated with 10% methanol and 90% water with 0.5% acetic acid at a flow rate of 50 mL/min, and the effluent was monitored at 280 nm. The column was developed to 100% methanol over 50 minutes. Fractions were collected based on time intervals and pooled based on peak separation. The procedure was repeated to obtain sufficient quantity of purified material. Fractions were allowed to evaporate to remove methanol and then freeze-dried to recover the purified fractions. Fractions were evaluated by HPLC and liquid chromatography-mass spectroscopy (LC-MS).

Analytical methodology. The HPLC analysis was conducted on a Shimadzu LC-20 HPLC system (LC-20AT

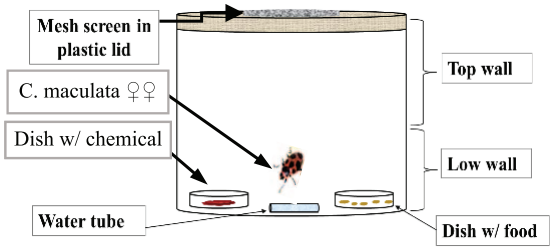

Figure 1. Plastic "test" cage to evaluate the effects of *Juniperus virginiana* fractions and synthetic bioflavonoids on *Coleomegilla maculata* oviposition site selection. Typical locations included low wall and top wall, dish with chemical, and dish with food. On occasion, a dish with nothing was included in bioassays. Control cages lacked the dish with chemical. Cage dimensions were 6-cm high × 8-cm diameter. Dish dimensions were 1.0-cm high × 3.5-cm diameter.

quaternary pump, DGU-20A5 degasser, SIL-20A HT autosampler, and a SPD M20A PDA detector) running under Shimadzu LC Solutions version 1.22 chromatography software (Columbia, MD, USA). The column used was an Inertsil ODS-3 reversed-phase C18 column (5 µ, 250 mm × 4.6 mm from Varian, Palo Alto, CA, USA). For phenolic compound analysis, the initial conditions were 20% methanol and 80% water with 0.05 M phosphoric acid, at a flow rate of 1 mL/min. The effluent was monitored at 325 nm on the variable wavelength detector. After injection (typically 25 µL), the column was held at the initial conditions for 2 minutes and then developed to 100% methanol in a linear gradient over 55 minutes.

LC-electrospray ionization-MS analysis for compound confirmation. Samples were run on a Thermo Electron LTQ Orbitrap Discovery Mass Spectrometer—a linear ion trap (LTQ XL) MS, coupled to a high precision electrostatic ion trap (Orbitrap) MS with a high-energy collision (HCD) cell—with an Ion Max electrospray ionization (ESI) source, and a Thermo Scientific ACCELA series HPLC system (ACCELA 1250 UHPLC pump, ACCELA1 HTC cool stack autoinjector, and a ACCELA 80-Hz PDA detector), all running under Thermo Scientific Xcalibur 2.1.0.1140 LC-MS software. The MS was typically calibrated at least weekly with a standard calibration mixture recommended by Thermo Scientific and the signal detection optimized by running the autotune software feature, as needed. The MS was run with the ESI probe in the negative mode. The source inlet temperature was 300°C, the sheath gas rate was typically set at 50 arbitrary units, the auxiliary gas rate was usually set at 5 arbitrary units, and the sweep gas rate was usually set at 2 arbitrary units. The maximal mass resolution was set at 30 000, the spray voltage was set at 3.0 kV, and the tube lens was set at −100 V. Other parameters were determined and set by the calibration and tuning process. For phenolic analysis, the initial solvent system was 10% methanol versus water with 0.25% formic acid at a flow rate of 0.25 mL/min. After injection (1 µL or less), the column was developed with a linear gradient to 100% methanol over 50 to 60 minutes. The column effluent was monitored at 280 and 340 nm in the PDA detector. The software package was usually set to collect mass data between 100 and 2000 AMUs. Generally, the most significant sample ions generated under these conditions were [M−1]− and [M+HCOO]−. Three mass spectrometry events were programmed to run in sequence in the MS: (1) LTQ(IT)-MS full scan *m/z* 150 to 2000, (2) LTQ(IT)-MS trap most abundant ion, then perform collision-induced dissociation at 35% energy, and (3) Fourier transform MS (FTMS) full scan *m/z* 150 to 2000. For the evaluation of Xcalibur accurate mass data by the Cerno BioScience LLC MassWorks 5.0.0.0 software, the FTMS was set to collect spectra at a resolution of 7500 and a range of *m/z* of 100 to 2000 and then evaluated by sCLIPS (self-calibrating line-shape isotope profile search) which enhances formula ID accuracy without the need to run calibration standards.

Purchase of synthetic bioflavonoids

Rather than using the limited quantity of bioflavonoids identified and purified from *J. virginiana* fractions (in this study), we decided to use synthetic bioflavonoids to evaluate their potential to stimulate *C. maculata* oviposition. We purchased synthetic bioflavonoids from Sigma-Aldrich (St. Louis, MO, USA) or MedChem Express LLC (Princeton, NJ, USA). We assumed that the synthetic bioflavonoids and those purified from *J. virginiana* fractions were essentially the same, in relation to chemical composition, volatility, and odor profile. Presumably, the behavioral responses of *C. maculata* females would not differ, regarding bioflavonoid source.

Ovipositional responses to J. virginiana fractions and synthetic bioflavonoids

We compared the ovipositional responses of *C. maculata* females confined to cages with or without fractions from *J. virginiana* heartwood sawdust, as well as synthetic bioflavonoids. Typically, *C. maculata* females require a pre-oviposition period of 2 to 3 weeks.[31] For each experiment, we harvested approximately 100 one-month-old adults (combined sexes) from the same generation (26th-29th generations) from our stock colony and

then subdivided them into 2 large containers (9.5-cm high, 11-cm diameter) for 2 to 3 days to ensure mating. Females were then isolated in a Petri dish with water (on a cotton wad) but no food, in a growth chamber for 24 hours, to standardize hunger levels among females destined for the bioassays.

We conducted bioassays in plastic cages (6-cm high, 8-cm diameter, 250-mL volume) with screened lids (Figure 1). A tissue paper (Kleenex) oviposition substrate was not used in test or control cages. Females were randomly selected from the large containers and placed separately into plastic cages, using a total of 7 replicate females in test and control cages, 1 female per cage. All cages were provisioned with an excess of factitious food (same food source reared on in the stock colony, ie, *E. kuehniella* eggs or *A. franciscana* eggs) and distilled water, in a small glass tube, at the base of the cage. Note that females were fed the identical type, and excess amount, of food in test and control cages within a given experiment. Test cages contained a tiny Petri dish (1.0-cm high, 3.5-cm diameter) with test chemical, 1 mg, *J. virginiana* fraction powder or synthetic bioflavonoid powder, at its base. Preliminary observations indicated that 1 mg of test powder was an adequate quantity to elicit oviposition behavior. No other concentrations were used in our bioassays. Because the test powder occasionally adhered to body parts of *C. maculata* females and their freshly laid eggs, we added more powder to the dish, as needed, to maintain approximately 1 mg in the dish at all times, during the course of the experiment.

Oviposition sites included the cage wall (lower wall and top wall; see Figure 1), dish with food, and dish with chemical in test cages. In control cages, oviposition sites were the same, but without the dish with chemical. Also, an empty Petri dish (1.0-cm high, 3.5-cm diameter) was in control cages in a few experiments (see Figure 2A and B; Figure 4B and C) to determine whether females were attracted to the dish alone. Twice each day, we recorded the location of egg clutches, the number of egg clutches, and the number of eggs in each clutch in test and control cages, for 12 to 13 consecutive days. Egg clutches were removed from cages as soon as they were seen, generally twice a day. All test and control cages were maintained in a climate-controlled growth chamber (24°C, 60% RH, 16 hours photophase) but removed twice daily to monitor oviposition behavior and record egg clutch data. Adult females were fed every other day; old food and waste (feces) were discarded.

Effects of synthetic bioflavonoid (quercetin) on egg hatch

We tested the effects of quercetin powder on egg hatchability. To set up the experiment, we harvested approximately 50 one-month-old adults (combined sexes) of the 37th generation from our stock colony and then subdivided them into 2 large containers (9.5-cm high, 11-cm diameter) for 2 to 3 days to ensure mating. Mated females were randomly selected from

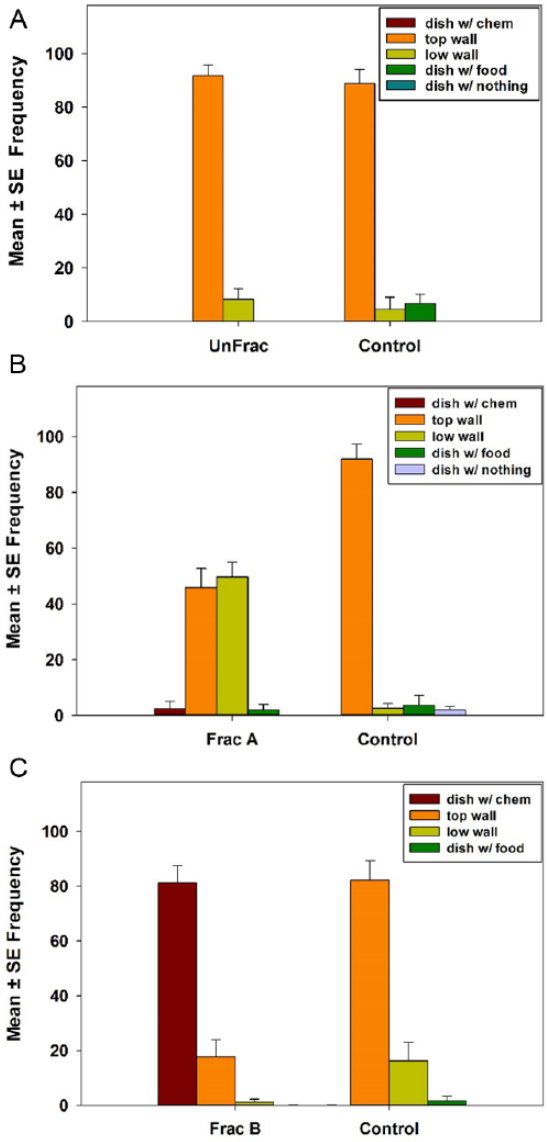

Figure 2. Effect of *Juniperus virginiana* (A) unfractionated powder, (B) fraction A powder, or (C) fraction B powder on mean (±SE) frequency of *Coleomegilla maculata* egg clutches at various locations in test and control cages (without compounds), pooled over consecutive days.

the containers and designated for experimental treatments. On the same day, 5 females were placed into 2 cages (6-cm high, 8-cm diameter, 250-mL volume) with tissue paper (oviposition substrate), and 5 females were placed in another cage (6-cm high, 8-cm diameter) with 1 mg of quercetin inside a tiny Petri dish (1.0-cm high, 3.5-cm diameter), at its base. Preliminary observations indicated that 1 mg of quercetin powder was an adequate quantity to elicit oviposition behavior. No other concentrations were used in this experiment. But note that quercetin powder occasionally adhered to body parts of *C. maculata* females and their freshly laid eggs. In this case, we added more powder to the dish, as needed, to maintain approximately 1 mg in the dish at all times, during the course of the experiment. All cages were provisioned with distilled water (in a centrifuge

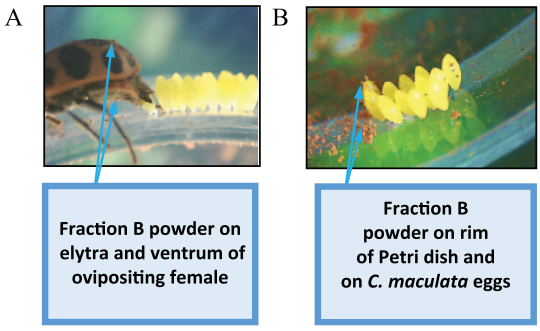

Figure 3. Image of (A) *Coleomegilla maculata* female ovipositing on the inner wall of a tiny Petri dish containing fraction B powder and (B) a *C. maculata* egg clutch on the inner wall of another dish containing the same compound. Dish dimensions were 1-cm high × 3.5-cm diameter.

tube, stoppered with a cotton wad) and *E. kuehniella* eggs, in excess. Egg clutches from one of the cages (designated as control) with tissue paper were cut out of the paper and transferred to a clean Petri dish (for hatching) each day. Egg clutches from the other cage with tissue paper were also cut out of the paper, transferred to a clean Petri dish (for hatching), but sprinkled with 1 mg quercetin powder, using a camel hair paint brush. For the last cage, the tiny Petri dish with quercetin powder was removed from the cage whenever eggs had been laid inside it and then placed inside an empty cage (for hatching). A replacement Petri dish was positioned in the test cage. Petri dishes and test cages were maintained in a climate-controlled growth chamber (24°C, 60% RH, 16 hours photophase) but removed twice daily, for 24 to 25 consecutive days, to record the number of egg clutches, number of eggs per clutch, hatch date, and the hatch rate in each clutch. Females were fed *E. kuehniella* eggs in excess, and uneaten food was discarded every other day. For this experiment, there were 43, 50, and 38 egg clutches from females in the control cage with tissue, cage with tissue and eggs sprinkled with quercetin, and the cage with quercetin in the tiny dish at its base, respectively.

Statistical analysis

Data were analyzed following a completely randomized design. A Pearson product-moment correlation analysis was used to test for a relationship between the frequency of egg clutches on the cage wall and in the dish with chemical. The Student's *t*-test compared the number of clutches (and the number of eggs per clutch) in test (fractions) versus control cages, regardless of location. The 1-way analysis of variance (1-way ANOVA) compared the number of clutches (and number of eggs per clutch) in test (bioflavonoid) versus control cages, regardless of location. The 1-way ANOVA also tested the effects of a bioflavonoid (quercetin) on proportional egg hatch. A Pearson product-moment correlation analysis was used to test for a relationship between *C. maculata* egg hatch rate and clutch size. Frequency (ie, percentage) data were arcsine transformed and absolute data were square root transformed when the assumptions of normality and equal variance were not met. When necessary, the Tukey-Kramer honest significant difference test was used as a multiple comparison procedure after the 1-way ANOVA. Statistical analyses were performed with SigmaStat 3.0.1 (interfaced through SigmaPlot 12, Systat Software Inc., in Richmond, CA, USA) and JMP 12.0.1 (2012 SAS Institute Inc., in Cary, NC, USA) computer software.

Results

Ovipositional responses to J. virginiana fractions

In bioassays using *J. virginiana* fraction powder, *C. maculata* females showed a preference for ovipositing at select "sites" in test or control cages (Figures 2 and 4). In test cages with unfractionated *J. virginiana* powder or fraction powder A, females preferred ovipositing on the top wall rather than anywhere else. No egg clutches were found in unfractionated *J. virginiana* test cages and hardly any were found in fraction A test cages (Figure 2A and B). There was a strong negative correlation between the frequency of egg clutches on the cage wall (top and low walls combined) and in the Petri dish with fraction A powder (Table 1). The preference for the top wall was also observed in control cages, which did not contain any *J. virginiana* fraction powder. In test cages with fraction B powder, females preferred to oviposit on the inner edge or rim of the Petri dish (ie, "dish with chem"), which held the powder (Figure 2C, Figure 3A and B). Nearly 80% of egg clutches were found in the dish over the course of this experiment. There was a strong negative correlation between clutch frequency on the cage wall (top and low walls combined) and in the Petri dish with fraction B powder (Table 1). In test cages with fraction C powder or fraction D powder, females preferred ovipositing in the dish containing the powder; slightly more than 80% of egg clutches were found in the dish (Figure 4A and B). In the control cage, associated with the fraction D bioassay, females oviposited equally well on the top wall and in the dish with food, at the base of the cage. In test cages with fraction E powder, females preferentially oviposited in the dish containing the powder (Figure 4C); slightly less than 80% of egg clutches were found in the dish. In the control, females tended to oviposit on the top and low walls of the cage. Note that strong negative correlations between clutch frequency on the cage wall (of test cages) and in the dish with chemical were also evident for fractions C, D, and E (Table 1).

Regardless of oviposition site, significantly more egg clutches (per day) were in test cages (rather than associated control cages) containing unfractionated extract, fraction B, fraction D, and fraction E (Table 2). Note that daily clutch number was often less than 1 in all experiments, except those involving fractions D and E. Clutch number was significantly greater in control cages (than in associated test

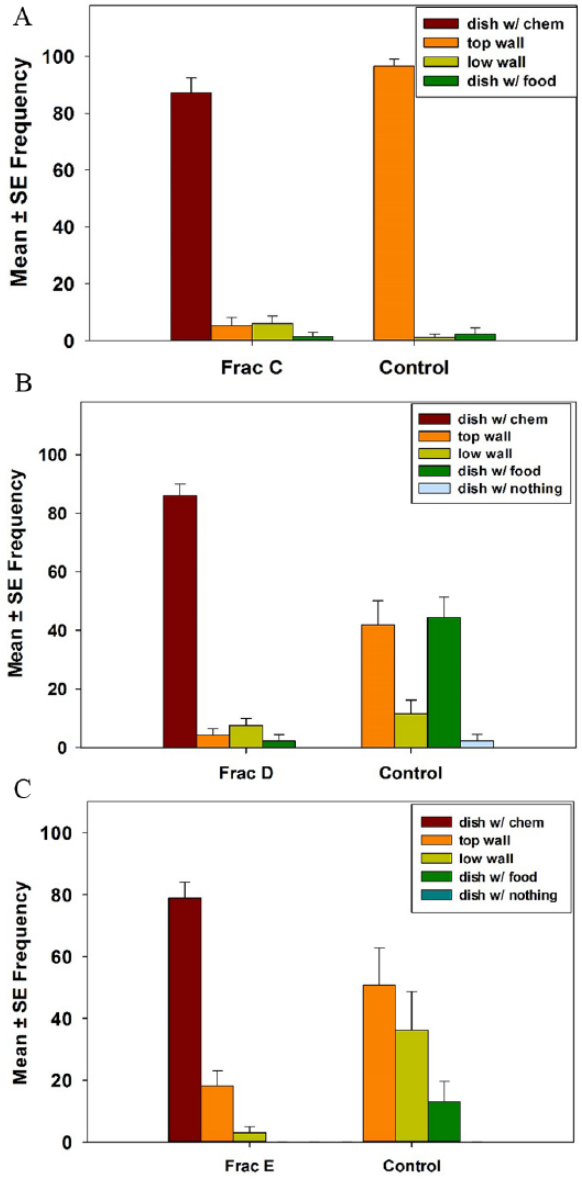

Figure 4. Effect of *Juniperus virginiana* (A) fraction C powder, (B) fraction D powder, or (C) fraction E powder on mean (±SE) frequency of *Coleomegilla maculata* egg clutches at various locations in test and control cages (without compounds), pooled over consecutive days.

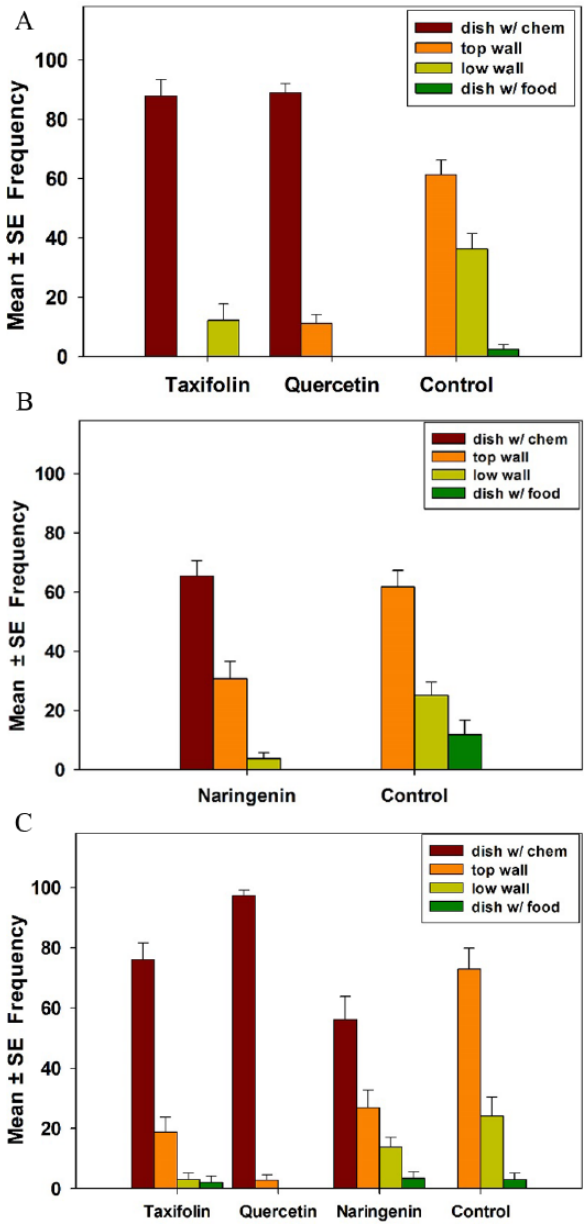

Figure 5. Effects of (A) taxifolin and quercetin, (B) naringenin, or (C) taxifolin, quercetin, and naringenin on mean (±SE) frequency of *Coleomegilla maculata* egg clutches at various locations in test cages and control cages (without compounds), pooled over consecutive days.

cages) in the experiment involving fraction A. When considering the number of eggs in a clutch (per day), significant differences between test and control cages were observed only in one experiment (Table 3). Control cages held significantly more eggs per clutch than test cages containing fraction C.

Identification of bioflavonoids in fractions

Chemical analysis revealed the presence of several bioflavonoids in *J. virginiana* heartwood fractions A, C, D, and E (Table 4). In fraction B, there was a complex of bioflavonoids (yet to be confirmed). Epicatechin and catechin were in fraction A, whereas taxifolin was in fractions C and D. Dihydrokaempferol was in fractions D and E; naringenin and quercetin were also in fraction E.

Ovipositional responses to synthetic bioflavonoids

As in the bioassays involving *J. virginiana* fractions, synthetic bioflavonoids affected the selection of oviposition sites by *C. maculata* (Figure 5). We present the data for 3 bioflavonoids: taxifolin, quercetin, and naringenin. In response to test cages

Table 1. Statistics for Pearson product-moment correlation analysis of mean frequency of egg clutches on cage wall (top and low walls, combined) and in dish with chemical (fraction powder).

VARIABLE	BY VARIABLE	CORRELATION (R_C)	N	*P* VALUE[a]
Unfrac.—on wall	Control—on wall	−0.18	11	.59
Unfrac.—in dish w/chem	Unfrac.—on wall	—	—	—
Frac. A—on wall	Control—on wall	−0.20	10	.58
Frac. A—in dish w/chem	Frac. A—on wall	−0.75	10	.012
Frac. B—on wall	Control—on wall	−0.25	10	.48
Frac. B—in dish w/chem	Frac. B—on wall	−1.0	10	<.0001
Frac. C—on wall	Control—on wall	−0.59	11	.056
Frac. C—in dish w/chem	Frac. C—on wall	−0.97	11	<.0001
Frac. D—on wall	Control—on wall	−0.38	9	.31
Frac. D—in dish w/chem	Frac. D—on wall	−0.82	9	.006
Frac. E—on wall	Control—on wall	0.52	9	.15
Frac. E—in dish w/chem	Frac. E—on wall	−1.0	9	<.0001

[a]Correlations are significant when $P < .05$. Refer Figures 2 and 4 for displays of actual frequency data.

Table 2. Mean ± SE number of *Coleomegilla maculata* clutches per day, regardless of oviposition site, in cages[1] with or without *Juniperus virginiana* powder.

TREATMENT	CLUTCHES PER DAY (MEAN ± SE)	*t*	*DF*	*P* VALUE	DAYS
Unfractionated	0.701 ± 0.071[a]	2.22	20	.038	12
Control	0.507 ± 0.052[b]				12
Fraction A	0.571 ± 0.060[b]	3.14	18	.006	11
Control	0.971 ± 0.112[a]				11
Fraction B	0.986 ± 0.086[a]	4.03	18	<.001	12
Control	0.557 ± 0.062[b]				12
Fraction C	0.792 ± 0.065[a]	0.91	20	.375	12
Control	0.909 ± 0.111[a]				12
Fraction D	1.063 ± 0.101[a]	4.95	16	<.001	12
Control	0.508 ± 0.048[b]				12
Fraction E	1.016 ± 0.060[a]	8.93	16	<.001	11
Control	0.365 ± 0.048[b]				11

t—statistic for Student's *t*-test. Mean values followed by a different letter denoted in superscript, in the same experiment, in a column, are significantly different ($P < .05$). On some days, no eggs were laid in either the test or the control cages.
[1]We used 7 replicate test and control cages with 1 mated female per cage.

with taxifolin or quercetin in the first experiment, females preferred to oviposit in the small dish containing either bioflavonoid (Figure 5A). More than 80% of daily egg clutches were found next to these 2 bioflavonoids over the course of this experiment. In control cages, females tended to prefer the top and low walls. In the second experiment, females preferentially oviposited in the small dish with naringenin rather than the top wall or other locations in test cages (Figure 5B). Slightly more than 60% of daily egg clutches were found next to naringenin. In the control cages, females preferred the top wall. In the third experiment, females preferred ovipositing in the small dish with taxifolin, quercetin, or naringenin rather than other locations in test cages. In this experiment, slightly less than 60% of daily egg clutches were next to naringenin, nearly 80% were next to

Table 3. Mean ± SE number of *Coleomegilla maculata* eggs per clutch per day, regardless of oviposition site, in cages with or without *Juniperus virginiana* powder.

TREATMENT	EGGS PER CLUTCH PER DAY[1] (MEAN ± SE)	*t*	*DF*	*P* VALUE	TOTAL CLUTCHES (7 FEMALES)
Unfractionated	13.85 ± 0.90[a]	1.76	92	.08	54
Control	16.47 ± 1.22[a]				40
Fraction A	11.05 ± 0.59[a]	0.29	106	.77	40
Control	11.29 ± 0.54[a]				68
Fraction B	13.54 ± 0.74[a]	0.15	106	.88	69
Control	13.36 ± 0.94[a]				39
Fraction C	12.03 ± 0.69[b]	2.36	129	.02	61
Control	14.43 ± 0.73[a]				70
Fraction D	12.36 ± 0.69[a]	0.25	97	.80	67
Control	12.66 ± 0.97[a]				32
Fraction E	8.78 ± 0.44[a]	0.99	85	.32	64
Control	9.78 ± 1.13[a]				23

t—statistic for Student's *t*-test. Mean values followed by a different letter denoted in superscript, in the same experiment, in a column are significantly different ($P < .05$).
[1]We used 7 replicate test and control cages with 1 mated female per cage.

Table 4. List of fractions from *Juniperus virginiana* heartwood and identification of some bioflavonoids and their properties.

FRACTION	BIOFLAVONOID	CHEMICAL FORMULA	IUPAC NAME[a]	MOLECULAR WEIGHT, G/MOL
A	Epicatechin	$C_{15}H_{14}O_6$	(2R,3R)-2-(3,4-dihydroxyphenyl) chroman-3,5,7-triol	290.271
	Catechin	$C_{15}H_{14}O_6$	(2R,3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol	290.271
B	Complex bioflavonoids	CHO?	N/A	N/A
C	Taxifolin	$C_{15}H_{12}O_7$	(2R,3R)-2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-2,3-dihydrochromen-4-one	304.254
D	Taxifolin	$C_{15}H_{12}O_7$	(As above)	(As above)
	Dihydrokaempferol	$C_{15}H_{12}O_6$	(2R,3R)-3,5,7-trihydroxy-2-(4-hydroxyphenyl)-2,3-dihydrochromen-4-one	288.255
E	Dihydrokaempferol	$C_{15}H_{12}O_6$	(As above)	(As above)
	Naringenin	$C_{15}H_{12}O_5$	5,7-dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydrochromen-4-one	272.256
	Quercetin	$C_{15}H_{10}O_7$	2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one	302.238

[a]National Center for Biotechnology Information. PubChem Compound Database; CID = 72276, https://pubchem.ncbi.nlm.nih.gov (accessed July 13, 2017).

taxifolin, but more than 95% were next to quercetin (Figure 5C). Note that strong negative correlations between clutch frequency on the cage wall (of test cages) and in the dish with chemical were evident for the bioflavonoids in all 3 experiments (Table 5).

Regardless of oviposition site, there were slightly more egg clutches in test cages than in control cages in 2 bioflavonoid experiments (Table 6). For example, significantly more egg clutches (per day) were observed in test cages (rather than associated control cages) in experiment 2, containing naringenin, and experiment 3, containing quercetin and naringenin. In experiment 3, the number of egg clutches was not different between cages with taxifolin versus control cages. In experiment 1, clutch number was not significantly different among taxifolin, quercetin, and the control cages (Table 6). Daily clutch number was often less than 1 in all experiments. When considering the number of eggs in a clutch (per day), significant differences between test and control cages were observed in experiment 3 (Table 7). Control cages held significantly

Table 5. Statistics for Pearson product-moment correlation analysis of mean frequency of egg clutches on cage wall (top and low walls, combined) and in dish with chemical (bioflavonoid powder).

EXPERIMENT	VARIABLE	BY VARIABLE	CORRELATION (R_C)	N	*P* VALUE[a]
1	Taxifolin—on wall	Control—on wall	0.27	13	.37
	Taxifolin—in dish w/chem	Taxifolin—on wall	–1.0	13	<.0001
	Quercetin—on wall	Control—on wall	–0.02	13	.95
	Quercetin—dish w/chem	Quercetin—on wall	–1.0	13	<.0001
2	Naringenin—on wall	Control—on wall	–0.22	12	.48
	Naringenin—in dish w/chem	Naringenin—on wall	–1.0	12	<.0001
3	Taxifolin—on wall	Control—on wall	0.14	12	.65
	Taxifolin—in dish w/chem	Taxifolin—on wall	–0.93	12	<.0001
	Quercetin—on wall	Control—on wall	–0.21	12	.51
	Quercetin—dish w/chem	Quercetin—on wall	–1.0	12	<.0001
	Naringenin—on wall	Control—on wall	0.16	12	.61
	Naringenin—in dish w/chem	Naringenin—on wall	–0.96	12	<.0001

[a]Correlations are significant when $P < .05$. Refer Figure 5 for displays of actual frequency data.

Table 6. Mean ± SE number of *Coleomegilla maculata* clutches per day, regardless of oviposition site, in cages[1] with or without bioflavonoid powder.

EXPERIMENT	TREATMENT	CLUTCHES PER DAY (MEAN ± SE)	*F*	*DF*	*P* VALUE	DAYS
1	Taxifolin	0.582 ± 0.068[a]	0.242	2, 36	.79	13
	Quercetin	0.648 ± 0.075[a]				13
	Control	0.615 ± 0.057[a]				13
2	Naringenin	0.964 ± 0.050[a]	21.81	1, 22	<.001	12
	Control	0.559 ± 0.062[b]				12
3	Taxifolin	0.774 ± 0.088[ab]	4.256	3, 44	.010	12
	Quercetin	0.821 ± 0.081[a]				12
	Naringenin	0.810 ± 0.059[a]				12
	Control	0.536 ± 0.050[b]				12

F—statistic for analysis of variance. Mean values followed by a different letter denoted in superscript, in the same experiment, in a column are significantly different (Tukey-Kramer test, $P < .05$).
[1]We used 7 replicate test and control cages with 1 mated female per cage.

more eggs per clutch than test cages, containing taxifolin, quercetin, or naringenin.

Effects of synthetic bioflavonoid on egg hatch

As in the case with *J. virginiana* fraction powder (eg, fraction B, see Figure 3B), bioflavonoid powder often adhered to the chorion of freshly laid *C. maculata* eggs. As expected, egg hatchability was unaffected by bioflavonoids, eg, quercetin. There were no significant differences in egg hatch rate when quercetin powder had been sprinkled on top of eggs, or when eggs had been oviposited directly in the small dish, already containing quercetin, or the control ($F_{2,128} = 1.38$; $P = .25$, Table 8). The mean hatch rate was 75% to 80%. But, clutch size differed significantly between the treatments ($F_{2,128} = 4.27$; $P = .016$, Table 8). Slightly more eggs were in clutches in the control (14.88 eggs per clutch) than in the quercetin in dish treatment (11.55 eggs per clutch); no differences were found between the control and quercetin sprinkled on eggs or between quercetin sprinkled on eggs and quercetin in dish treatments. Clutch size and hatch rate were not correlated for the control ($R_c = 0.237$, $P = .127$, n = 43), quercetin sprinkled on eggs ($R_c = .234$; $P = .102$; n = 50), or quercetin in dish ($R_c = 0.085$; $P = .612$; n = 38) treatments in this experiment.

Table 7. Mean ± SE number of *Coleomegilla maculata* eggs per clutch per day, regardless of oviposition site, in cages[1] with or without bioflavonoid powder.

EXPERIMENT	TREATMENT	EGGS PER CLUTCH PER DAY (MEAN ± SE)	*F*	*DF*	*P* VALUE	TOTAL CLUTCHES (7 FEMALES)
1	Taxifolin	11.55 ± 0.85[a]	0.47	2, 165	.63	53
	Quercetin	12.00 ± 0.64[a]				59
	Control	11.14 ± 0.64[a]				56
2	Naringenin	14.11 ± 0.70[a]	3.20	1, 126	.076	81
	Control	12.00 ± 0.66[a]				47
3	Taxifolin	11.52 ± 0.52[b]	8.57	3, 243	<.001	65
	Quercetin	10.23 ± 0.42[b]				69
	Naringenin	10.18 ± 0.50[b]				68
	Control	14.02 ± 0.84[a]				45

F—statistic for analysis of variance. Mean values followed by a different letter denoted in superscript, in the same experiment, in a column are significantly different (Tukey-Kramer test, $P < .05$).
[1]We used 7 test and control cages with 1 mated female per cage.

Table 8. Mean ± SE number of eggs per clutch and hatch rate for bioassay to test the effect of quercetin powder on *Coleomegilla maculata* egg hatchability.

TREATMENT	EGGS PER CLUTCH PER DAY (MEAN ± SE)	PROPORTIONAL HATCH RATE (MEAN ± SE)	TOTAL CLUTCHES
Control	14.88 ± 1.02[a]	0.763 ± 0.028[a]	43
Quercetin sprinkled on eggs	13.92 ± 0.73[ab]	0.753 ± 0.028[a]	50
Quercetin in dish	11.55 ± 0.87[b]	0.809 ± 0.029[a]	38

Mean values followed by a different letter denoted in superscript in a column are significantly different ($P < .05$, Tukey-Kramer test, following analysis of variance).

Discussion

In this study, the observation that *J. virginiana* fractions (with the exception of fraction A) modified the selection of oviposition sites by *C. maculata* females is reported herein and confirms previous research published by Boldyrev et al[33] and Smith et al.[34] In their laboratory bioassays, they used methanol-based *J. virginiana* fractions to elicit oviposition behavior in *C. maculata* as well as other ladybirds (*A. bipunctata, C. transversoguttata*, and *C. munda*). This study represents an advancement in this line of research because specific bioflavonoids were identified in *J. virginiana* fractions and several of these stimulated oviposition in *C. maculata*.

The observation that *J. virginiana* unfractionated powder and fraction A powder did not stimulate oviposition in *C. maculata* females could suggest that the concentration of phenolics in this fraction was rather low. Although we did not measure phenolic content, this fraction probably consisted mostly of carbohydrates and peptides rather than phenolics (M.A.B., unpublished data). Likewise, we did not quantify the concentration of bioflavonoids identified in fraction A, ie, epicatechin and catechin. Also, we have not tested either of these compounds, to date, to see whether they repel or attract ladybirds (or stimulate oviposition). But we have observed that a hydrated form of catechin (catechin hydrate) was attractive and stimulated *C. maculata* females to deposit 80% of their egg clutches near catechin hydrate powder rather than any other location inside test cages (E.W.R. and Z.W., unpublished data). Follow-up experiments are necessary to clearly determine whether either epicatechin or catechin inhibit or stimulate *C. maculata* oviposition. All other fractions (B, C, D, and E) stimulated oviposition in *C. maculata*, suggesting that phenolic (or bioflavonoid) content was moderate to high in these fractions.

Other bioflavonoids identified in *J. virginiana* fraction powder, such as dihydrokaempferol (in fractions D and E), were not included in our bioassays in this study. Interestingly, a closely related bioflavonoid, kaempferol, did not stimulate *C. maculata* females to oviposit in test cages (E.W.R. and Z.W., unpublished data). Yet, whether or not dihydrokaempferol would elicit the same negative oviposition response in *C. maculata* is unknown and warrants further study.

The observation of significantly more egg clutches in the vicinity of *J. virginiana* fraction powder B, D, and E as well as the unfractionated extract in test cages, in comparison with the control cages, suggests that phenolic compounds have a physiological effect on females, stimulating them to generate more egg clutches. Also, naringenin and quercetin (both identified in

fraction E) appeared to have a similar effect on *C. maculata*; females oviposited more egg clutches in test cages with either of these compounds than in control cages. The exact mechanism responsible for stimulating the production of more egg clutches in the presence of some bioflavonoids, and not others, is unknown. Although the number of egg clutches increased on occasion, the number of eggs in a clutch rarely ever increased in the presence of fractions or bioflavonoids in test cages, in comparison with control cages. A simple explanation for this observation is that females are limited in the number of eggs they can produce over time because of the rate at which eggs mature in their ovaries. Egg maturation rate is probably more dependent on a physiological response to ambient temperature, food quality, and ovariole size in ladybirds.[31,43]

Although our bioassays were not specifically designed to compare capacity to stimulate *C. maculata* oviposition between different bioflavonoids, our data suggest that quercetin and taxifolin were slightly more effective than naringenin. We do not have an explanation for these apparent differences between bioflavonoids but the molecular structures could provide clues. Naringenin is a smaller molecule than taxifolin and quercetin; it also contains 2 fewer oxygen atoms (see Table 4). There could be a connection between the number of oxygen atoms (as well as the number of hydrogen atoms) in these molecules with their capacity to stimulate oviposition in ladybirds, eg, *C. maculata*. Also, quercetin is classified as a flavonol, whereas taxifolin and naringenin are flavanones. Whether or not the classification has any bearing on stimulating oviposition is unknown, to our knowledge.

Maybe when the oxygen atoms in the quercetin and taxifolin molecules are exposed to air, they release odors, as they undergo biodegradation. Perhaps, these molecules have a faint odor (fragrance), which resembles the fragrance of plant foliage under attack by herbivorous insects, eg, aphids. These odors could trigger an innate response in *C. maculata* females, causing them to oviposit near quercetin and taxifolin, as if a natural aphid-infested leaf was present. Ladybirds usually oviposit on aphid-infested plants under natural field conditions.[20,21]

Monarch butterflies, which are deemed beneficial because they pollinate plants, use flavonols, such as quercetin, as cues for oviposition on host plants in the genus *Asclepias*.[39,44] Flavonols are present on the leaf surface, regardless of whether the plant is infested with pestiferous insects. Using the sensilla on the antennae and mid-tarsi, females of the monarch *Danaus plexippus* L. (Lepidoptera: Nymphalidae) detect quercetin (ie, quercetin glycosides) on the leaf surface. Taste sensilla on the fore-tarsi were not involved in recognition of quercetin.[39] Thus, *D. plexippus* females do not have to taste or ingest quercetin before oviposition stimulation ensues.

Adult females of the adzuki bean weevil *Callosobruchus chinensis* L. (Coleoptera: Bruchidae) are attracted to flowers of their host plant, adzuki bean *Vigna angularis* (Wild.) Ohwi and Ohashi.[45] Females use catechin and taxifolin as oviposition cues to locate young and immature *V. angularis* pods.[45,46] If these 2 bioflavonoids are present on the surface of host flowers and leaves, *C. chinensis* females do not need to feed on host plants (or bore into bean pods) to elicit oviposition stimulation.

Chemoreceptors on the mouthparts of *C. maculata* females could play a significant role in detecting tasteful or distasteful molecules in the *J. virginiana* fractions and bioflavonoids, thus inhibiting or stimulating oviposition in this beneficial insect. We often observed females "tasting" the fraction powder and bioflavonoid powder while inside test cages. We did not measure the quantity of powder they might have "tasted" over the course of an experiment. But females do ingest some of the powder because we have seen it in their feces (Z.W., unpublished data).

Despite the fact that some of the fraction powder adheres to the chorion of the eggs, no negative effects are apparent on hatch rate. This suggests the harmless nature of the molecules via physical contact and that fraction powder does not prevent the eclosion of first instars from the egg chorion. We did not report data on larval survival, posthatching, in this study. But our unpublished data indicate that *J. virginiana* fraction powder, or synthetic bioflavonoid powder, has no negative effects on *C. maculata* larval development.

We documented the ovipositional responses of individual females to *J. virginiana* fractions and synthetic bioflavonoids in cage bioassays. Although we show that most of the fractions and bioflavonoids, evaluated in this study, have promise as attractants and oviposition stimulants for *C. maculata* individually, we must test the effects of these molecules on *C. maculata* while in the company of conspecifics. Any cost-effective rearing system designed for ladybirds will involve adult females for a certain period of time in oviposition chambers, in the company of conspecifics, to conserve space and resources. However, females may or may not lay eggs under these conditions, and if they do in fact lay eggs, they could be inclined to cannibalize the newly laid eggs of conspecific females. This next step in our research on using bioflavonoids to boost *C. maculata* oviposition in the company of conspecifics, in rearing systems, is currently ongoing.

Conclusions

Mass-reared *C. maculata* females preferentially oviposited within 1 to 2 cm of most *J. virginiana* fractions. Chemical analysis identified bioflavonoids in most heartwood fractions and subsequent bioassays confirmed that quercetin, taxifolin, and naringenin (to a lesser extent) attracted females and stimulated oviposition. Females often laid more egg clutches in test cages than control cages. Quercetin powder often adhered to the chorion of freshly laid eggs but did not reduce hatch rate. Hatch rate was not correlated with clutch size. In this initial study, we show the potential benefits of several bioflavonoids on *C. maculata* oviposition in the laboratory with possible implications for using them to boost oviposition in mass rearing systems.

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Author Contributions

EWR, ZW, and FJE conceived and designed the experiments. EWR analyzed the data and wrote the first draft of the manuscript. EWR, ZW, FJE, and MAB contributed to the writing of the manuscript and agree with manuscript results and conclusions. EWR, FJE, and MAB jointly developed the structure and arguments for the paper, made critical revisions, and approved final version. All authors reviewed and approved the final manuscript.

Disclosures and Ethics

The authors, E.W.R., Z.W., F.J.E., and M.A.B. have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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