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# The Production of Sex Pheromone in Lady Beetles Is Conditioned by Presence of Aphids and Not by Mating Status

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## Abstract

Female *Harmonia axyridis* (Coleoptera: Coccinellidae) produce a sex pheromone to attract males. In the present study, we tested two hypotheses: (i) production of sex pheromone in *H. axyridis* is conditioned by perception of prey in their vicinity, and (ii) virgin females invest in the production of the sex pheromone, while mated females reduce their pheromone emissions. To test the first hypothesis, newly hatched larvae were divided into three groups: those fed with *Ephestia kuehniella* eggs, those fed with pea aphids, and those exposed to aphid volatile cues but fed with *Ephestia* eggs. All females produced a pheromone blend of five-components in similar relative proportions, but with contrasting absolute quantities: Females fed with *Ephestia* eggs produced lower amounts of pheromone ($0.5 \pm 0.4$ ng.female$^{-1}$), compared to females fed with aphids ($44.2 \pm 24.4$ ng.female$^{-1}$). The females of the third group produced intermediate concentrations of pheromone ($6.0 \pm 3.2$ ng.female$^{-1}$). To test the second hypothesis, two groups of lady beetles were made: one group of females was placed in the presence of males, while females of the other group were not. Mated and virgin females produced statistically similar amounts of pheromone ($144.1 \pm 49.7$ ng and $43.7 \pm 24.1$ ng.female$^{-1}$, respectively). These results suggest that *H. axyridis* females initiate pheromone production upon exposure to volatile cues released by their aphids prey. Females continue to release sex pheromone after mating, probably to increase the chance of multiple mating which is known to improve egg fertility.

**Keywords** *Harmonia axyridis* · Chemical signaling · Semiochemical · Effect of diet · Coccinellidae · Pea aphids · Sexual selection

## Introduction

The multicolored Asian lady beetle, *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae), is an invasive species that has been introduced into North America, Europe and Russia (Roy et al. 2016). Among the behavioral and immunological features that may have helped this species to become so successful is use of a complex semiochemical-based communication system governing sexual interactions (Fassotte et al. 2016; Legrand et al. 2019a, b; Sloggett et al. 2011; Verheggen et al. 2017). Like other lady beetle species, adult *H. axyridis* go through a maturation period of their sexual organs, which starts directly after emergence (Obata and Hidaka 1987). Once sexually mature, *H. axyridis* females display a stereotyped calling behavior: they raise their elytra and squeeze their abdomen, but only in the presence of prey (Fassotte et al. 2014). Males of related species were shown to be attracted by females over a distance (Schaller and Nentwig 2000; Omkar and Pervez 2005), and Fassotte et al. (2014) identified a volatile sex pheromone in *H. axyridis*, consisting of a mixture of five compounds: (−)-β-caryophyllene, α-humulene, α-bulnesene, methyleugenol and β-elemene, in descending order of relative proportions. This female pheromone elicited attraction of males, that perceived the signal by means of trichoid sensilla (Chi et al. 2009).

In the present study, we raised two hypotheses. According to the first, the production of sex pheromone in *H. axyridis* is conditioned by the presence of prey. We based this first hypothesis on the observation of Fassotte et al. (2014) that “three days after the lady beetles consumed aphids, they rhythmically extended and contracted their abdomens, while raising their elytra above their bodies”. Feeding on aphids could provide the female with the precursors necessary for the synthesis of the pheromone (Landolt and Phillips 1997). That the diet

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impacts pheromone production and composition is documented in several insect Orders, including other Coleopterans such as *Tenebrio* beetles (Rantala et al. 2003) and burying beetles (Chemnitz et al. 2015). However, these studies were mainly focused on male sex pheromones, and tested the link between the diet quality, the signaler's fitness and the chemical composition of the pheromone (Darragh et al. 2019).

We also tested a second hypothesis that virgin females invest in the production of the sex pheromone, while mated females reduce their pheromone emissions. Females must reach a trade-off between the costs of producing pheromone and the attraction of additional males. Lady beetle females benefit from multiple matings as repeated copulations with different males increase the egg fertility of females (Bayoumy and Michaud 2014; Majerus 1994). Thus release of pheromone after mating, but in smaller quantities, could be valuable, especially if the cost of production is low, which is likely to occur if the pheromone precursors are provided in the diet.

## Methods and Materials

### Insect Rearing

The initial population of *H. axyridis* (approximately 200 individuals) was collected as adults in the field in Gembloux (Belgium) during summer 2019. They were kept in aerated plastic boxes (36 × 15 × 8 cm; 5 males and 15 females per box), placed in a climate-controlled room (16:8 L:D photoperiod, 50 ± 10% RH, 24 ± 1 °C) and were provided with pea aphids, *Acyrthosiphon pisum* Harris, reared on *Vicia faba,* to induce mating and oviposition. Eggs were collected by introducing folded filter paper (90 mm diameter) into the box. Egg patches (<20 eggs) were individually placed in Petri dishes (4 cm diameter; 1 cm height).

### Impact of Diet

Newly-hatched *H. axyridis* larvae were grouped in aerated plastic boxes (36 × 15 × 8 cm; 20 larvae per box) containing a water-impregnated sponge. Three types of diets were provided during the entire larval development, as well as during the first 20 days of adult life. Individuals of the first group were fed ad-libitum with frozen *Ephestia kuehniella* Zeller eggs, while those in the second group were fed ad-libitum with pea aphids. Individuals of the third group were exposed to the smell of a pea aphid colony, but were fed ad-libitum with *E. kuehniella* eggs. To do so, a Petri dish (5 cm dia) closed with a grid (fine mesh) and containing approximately 2 g of aphids was introduced into the box. Aphids were feeding on a faba bean leaflet, kept fresh by a thin layer of agar poured into the bottom of the box, and the petri dish with aphids was replaced every two days. This prevented the beetles accessing the aphids while exposing them to their odorant cues.

Adult males were discarded after hatching. The sex pheromone was collected from individual virgin females aged of 20 days (12 females fed with pea aphids, 11 females fed with *E. kuehniella* eggs, 11 females exposed to pea aphid cues but fed with *E. kuehniella* eggs). This duration was chosen to ensure that all analyzed females reached sexual maturity (Obata and Johki 1991) and were likely to release sex pheromone (Fassotte et al. 2014).

### Impact of Mating Status

In the second experiment, newly emerged larvae were grouped in aerated plastic boxes (36 × 15 × 8 cm; 20 larvae per box) and were fed ad-libitum with pea aphids. Adults were sexed after emergence, before being distributed into six aerated plastic boxes. Three boxes contained 10 males and 10 females, and the other three contained 20 females. Adults from both groups were fed with pea aphids until aged 20 d when sex pheromone was collected from females. This duration was chosen to ensure that all analyzed females reached sexual maturity (Obata and Johki 1991) and were likely to release sex pheromone (Fassotte et al. 2014). Four female beetles were randomly selected from each box and pheromone collected from individual beetles. In subsequent analyses, two samples were rejected due to technical issues to give $N = 10$ mated females and $N = 12$ virgin females.

### Pheromone Collection

Pheromone collection was performed using a glass Y tube (h = 10 cm; d = 1 cm) into which a single female was introduced. The glass tube was immersed in a Bain Marie at 35 °C, to increase volatilization of the compounds. An air pump pulled charcoal-filtered air through the Y tube at 150 $\text{ml.min}^{-1}$, and the lady beetle was crushed during 3 min using a magnetic stirrer bar, as described in Fischer and Lognay (2012). Volatile organic compounds were trapped during 30 min in a Tenax® TA thermodesorption tube (Markes International Ltd., Llantrisant, UK). This duration was previously found to be sufficient to collect all VOCs produced by a single lady beetle, without experiencing breakthrough. After sampling, all tubes were stored at 4 °C before analysis.

### Pheromone Analysis

All samples were analyzed by gas chromatography coupled to mass spectrometry (GC-MS:GCMS_QP 2020 NX, Shimadzu, Kyoto, Japan). Volatiles were desorbed at 280 °C during 8 min, before being trapped by Peltier effect at −20 °C and heated at 280 °C to be injected on a capillary column (5% diphenyl; 30 m × 0.25 mm I.D.; film thickness 0.25 μm). A split ratio of three was applied, and helium was used as carrier gas constant flow rate of 1.0 ml.min$^{-1}$. The oven temperature program was initiated at 40 °C, held for 2 min, raised at 8 °C.min$^{-1}$ to 250 °C, and raised at 20 °C.min$^{-1}$ to 320 °C. The final temperature was held for 3 min. Mass spectra were recorded from 4 min after the start of injection until the end of the temperature program. The electron impact mode was set at 0.1 kV (source at 200 °C, transfer line at 230 °C, scanned mass range: 30 to 350 m/z). Components detected were identified by interpretation of their mass spectra and comparison with spectral libraries (NIST and FFNSC). Retention indices were determined using the retention times of *n*-alkane standards (from C4 to C30, 10 mg/ml in *n*-hexane), and compared with retention indices provided in the libraries. The identification of volatile compounds of the sex pheromone was then confirmed by injecting commercial standards (98% purity): β-caryophyllene, β-elemene, α-humulene and methyleugenol (Sigma Aldrich). All compounds were quantified using n-butylbenzene (Sigma Aldrich) as an internal standard (8.5 ng/μl).

### Statistical Analyses

Volatile profiles were compared using a permutational multivariate analysis of variance (perMANOVA), using a Euclidian distance matrix and 999 permutations (“adonis” command, vegan R-package). One-way ANOVA were also performed on each component of the sex pheromone to describe differences among treatments of diets and mating status. A logarithmic transformation was applied to meet the conditions of normality and homoscedasticity in both uni- and multivariate analyses. All statistical analyses were performed on peak areas.

## Results

### Impact of Diet

We were able to quantify four of the five compounds previously identified by Fassotte et al. (2014) in the female sex pheromone of *H. axyridis*: when β-elemene was detected, it was below the limit of quantifications (Fig. 1). β-Elemene was detected only in the lady beetles fed with aphids and the lady beetles fed with *Ephestia* eggs and exposed to aphid volatile cues. The total amount of pheromone was impacted by the diet ($F_{2,31} = 3.17$; $P = 0.001$), with 44.2 ± 24.4 ng, 0.5 ± 0.4 ng, and 6.0 ± 3.2 ng for lady beetles fed with aphids, fed with *Ephestia* eggs, and fed with *Ephestia* eggs but exposed to aphid cues, respectively. All components were produced in significantly smaller amounts when *Ephestia* eggs were used as a diet instead of aphids (Fig. 1). The relative proportions of each pheromone components were similar, whatever the diet used. Table 1 shows the relative proportions of the five components released by female lady beetles fed with aphids, as reported by Fassotte et al. (2014), Legrand et al. (2019c) and the present study. No obvious differences were apparent in these three studies: (−)-β-Caryophyllene was the major component (60–85%), followed by α-humulene, α-bulnesene, methyleugenol, and β-elemene.

### Impact of Mating Status

β-Elemene was detected in most individuals but below the limit of quantification (Fig. 2). The total amount of pheromone was not impacted by the mating status of the female ($F_{1,20} = 2.08$; $P = 0.1$) with 43.7 ± 24.1 ng and 144.1 ± 49.7 ng, from virgin and mated females, respectively. The mating status did not impact the relative proportions of the four components of the pheromone, but the absolute quantity of each component was increased in mated individuals, reaching statistical significance for methyleugenol and bulnesene (Fig. 2; methyleugenol $F_{1,20} = 7.254$, $P = 0.014$; α-bulnesene $F_{1,20} = 7.60$, $P = 0.012$;, β-caryophyllene $F_{1,20} = 3.90$, $P = 0.062$; α-humulene $F_{1,20} = 0.80$, $P = 0.382$).

## Discussion

Fassotte et al. (2014) reported the existence of a volatile sex pheromone in the lady beetle species, *Harmonia axyridis*. The presence of the same five pheromone components was subsequently observed in females originating from China, Europe and North America (Legrand et al. 2019c), in similar relative proportions among the tested populations, but with contrasting absolute quantities. In the present work, we also found the same gender-specific volatile blend: All five compounds previously reported were detected, although β-elemene was only present in trace amounts. All three studies (Fassotte et al. 2014, Legrand et al. 2019c and the present study) reported comparable relative proportions of the five components with (−)-β-caryophyllene the major component followed by α-humulene, α-bulnesene, and methyleugenol. In this study, the relative proportion of (−)-β-caryophyllene found was lower than in the previous studies (reduced from 83 to 85 to 63%) with a corresponding increase in the relative amount of α-humulene (from 8 to 9 to 31%). Differences in absolute quantities observed in these three reports are probably due to differences in the methodologies for volatile samplings. In the present study, we decided to crush the beetles for two main reasons: The objective of the present study was to test the impact of treatments on pheromone production, and not pheromone emission, and analyzing the volatile content of crushed individuals was likely to lead to less variability among

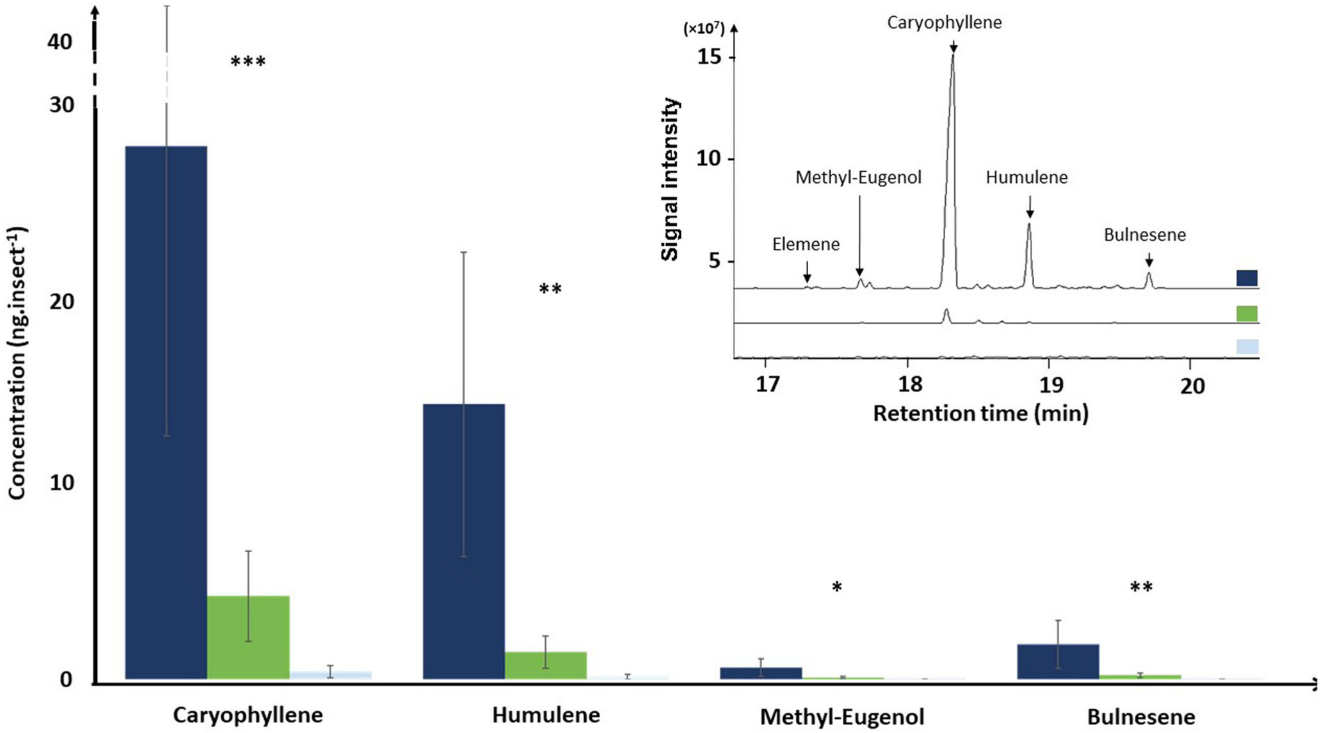

**Fig. 1** Impact of diet on the production of components of the sex pheromone of *Harmonia axyridis*. Dark blue: lady beetles fed with aphids, green: lady beetles fed with Ephestia eggs in presence of aphid volatile cues; light blue: lady beetles fed with Ephestia eggs in absence of aphids. Top right three total ion chromatograms of the lady beetle pheromone under the same three diets. β-Caryophyllene $F_{2,31} = 10.29$; $P < 0.001$; α-humulene $F_{2,31} = 7.89$; $P = 0.002$; methyleugenol $F_{2,31} = 4.41$; $P = 0.021$; α-bulnesene $F_{2,31} = 7.41$; $P = 0.002$

replicates than using living individuals. Indeed, we suspect the emission of pheromone to be affected by other factors, such as the hour of the day.

Lady beetles fed with *Ephestia* eggs produced a sex pheromone of similar composition to that from beetles fed on aphids, but in lower absolute amounts, with 99% reduction in absence of aphid volatile cues and 86% reduction in presence of aphid volatile cues. Thus it seems unlikely that aphid-specific precursors are required for pheromone biosynthesis, and more likely that aphids promote the production of sex pheromone rather than being a prerequisite. It is possible that lady beetles fed only *Ephestia* eggs may have a lower fitness than those fed aphids, and this could be associated with a reduction in pheromone production. However, our results indicate a volatile cue is released by aphids and perceived by lady beetles, informing them about the presence of prey, and triggering the biosynthesis of the sex pheromone. Because aphids were introduced in the lady beetle rearing with a plant leaflet, we cannot exclude the potential role of herbivore-induced plant volatiles (HIPV) (Takemoto and Takabayashi 2015). However, we think HIPVs would not make reliable cues: they vary among plant species, they can be impacted by various biotic and abiotic factors, and are not always aphid-specific (Verheggen et al. 2013).

**Table 1** Comparison of relative proportions of components of the female sex pheromone in *Harmonia axyridis* reported in previous literature and the present study

| Pheromone components | Relative proportions (%) | | |
|---|---|---|---|
| | Fassotte et al. 2014[a] | Legrand et al. 2019c[b] | Present study[c] |
| β-Elemene | 1.5 | 1.2 | 0 |
| Methyleugenol | 2.6 | 2.3 | 1.4 |
| (−)-β-Caryophyllene | 83.4 | 85.4 | 63.1 |
| α-Humulene | 9.3 | 8.4 | 31.5 |
| α-Bulnesene | 3.2 | 2.7 | 4.1 |

[a] European strain of lady beetles fed with aphid for 15 d

[b] Chinese strain of lady beetles fed with aphid for 15 d

[c] European strain of lady beetles fed with aphid for 20 d

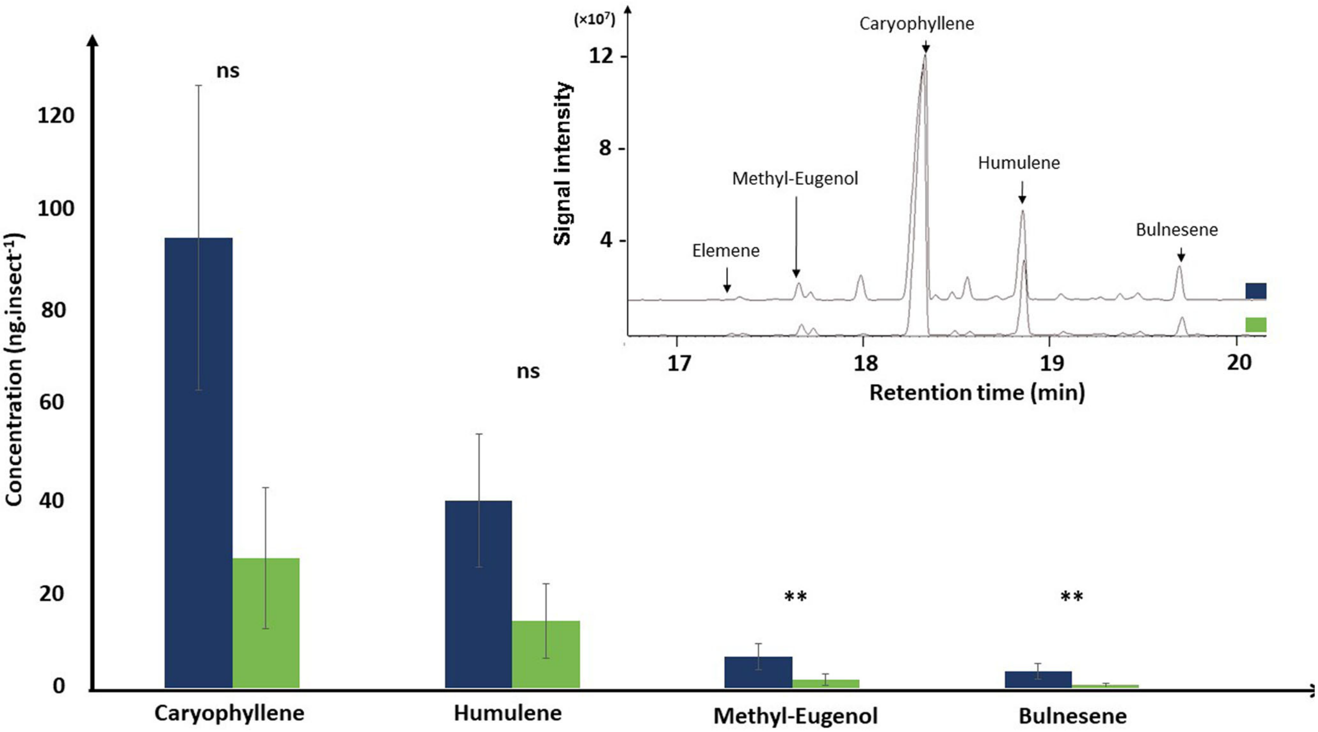

**Fig. 2** Impact of mating status on the production of components of the sex pheromone of *Harmonia axyridis*. Dark blue: mated lady beetles, green: virgin lady beetles. Top right two total ion chromatograms of the lady beetle pheromone under the two mating status (* indicates means significantly different $P < 0.05$)

Aphids are known to be the source of different volatile molecules, and many of them are already known to attract *H. axyridis* females and to allow them to assess the fitness of an aphid colony (Osawa 2000; With et al. 2002). Lady beetles could use one of two aphid-associated volatile cues: honeydew volatiles or aphid alarm pheromone. Honeydew is a sugar-rich water solution deposited on the plant tissue by aphids while feeding. It is colonized by bacteria that are responsible for honeydew-associated volatile cues (Leroy et al. 2011). Honeydew volatiles are used as kairomones for aphid predators such as lady beetles (Leroy et al. 2012). More than 15 compounds are typically released, and each of them, alone or in combination, may inform lady beetles of the presence of prey. Aphids were feeding on a plant leaf when introduced in the lady beetle rearing and were likely to have produced honeydew. The sesquiterpene, (*E*)-β-farnesene acts as an alarm pheromone in aphids (Boullis and Verheggen 2016; Kislow and Edwards 1972). It is typically released by aphids under predation but is also released continuously, in smaller quantities, in absence of predator (Almohamad et al. 2008; Verheggen et al. 2008). Like honeydew volatiles, (*E*)-β-farnesene acts as a kairomone for lady beetles, guiding adults and larvae toward their prey (Acar et al. 2001; Sloggett et al. 2011; Verheggen et al. 2007, 2009).

Based on our previous results on aphid pheromone production (Verheggen et al. 2009), we hypothesize that (*E*)-β-farnesene is the cue used by lady beetles. Indeed, pea aphids (*Acyrthosiphon pisum*) reared in isolation were found to produce significantly lower alarm pheromone than individuals reared among conspecifics. Also, pea aphids reared in isolation but exposed to (*E*)-β-farnesene, produced similar quantities of pheromone to individuals reared among conspecifics (Verheggen et al. 2009). Additional work is needed to identify which compound(s) is perceived and induce the production of sex pheromone in female lady beetle. Analyzing the pheromone content of newly emerged lady beetles exposed to dispensers releasing aphid-specific compounds would provide valuable information, as would exposing lady beetles to aphids before switching to *Ephestia* eggs for several days and checking for possible reduction in pheromone production. It is important for the larvae to hatch close to an aphid colony because they have limited dispersal abilities (Dixon 2000), although, if mating occurs in absence of prey, *H. axyridis* females can still store the spermatozoa acquired during copulation in their spermatheca for a period long enough to locate a colony of aphids (Awad et al. 2013).

The second hypothesis tested in this study was that virgin females produce higher amounts of sex pheromone than mated ones. However, we found no difference in the absolute quantity of sex pheromone between virgin and mated females. Although mated beetles produced three times more pheromone than virgin ones, these differences were not statistically significant due to the variability in emissions from tested females. This is consistent with the fact that females of *H. axyridis* are highly polyandrous. They not only mate multiple times but also use the sperm from multiple males to fertilize their eggs (Awad et al. 2015). Repeated copulations with different males increase egg fertility (Majerus 1994; Bayoumy and Michaud 2014). We have no information related to pheromone biosynthesis, but one can conclude that the cost of pheromone production is likely to be lower than the benefit of having multiple partners.

Based on the data of the present work, we conclude that females of *Harmonia axyridis* condition pheromone production to the presence of prey and use volatile cues released by aphids to initiate pheromone biosynthesis. Once mated, a female continues to release sex pheromone to increase her chance to have multiple matings.

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**Authors' Contributions** AC and CM performed the experiments and analyzed the data. FV conceived the methodologies and wrote the manuscript.

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**Data Availability** All data are available upon request.

## Compliance with Ethical Standards

**Conflicts of Interest/Competing Interests** The authors declare no conflict of interest or competing interests.

**Ethics Approval** Non applicable.

**Consent to Participate** Non applicable.

**Consent for Publication** The authors consent on publication.

**Code Availability** Non applicable.

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