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## Cuticular hydrocarbon composition does not allow *Harmonia axyridis* males to identify the mating status of sexual partners

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With 2 figures and 2 tables

**Abstract:** Males of polyandrous species have to overcome sperm competition. They should select their mate based on the reproductive status of the female to increase their own fitness. Because the sexual behavior of lady beetles relies on semiochemicals, with cuticular hydrocarbons (CHCs) being used for mate recognition, we developed and tested two hypotheses. First, we hypothesized that the cuticular hydrocarbon profile qualitatively and quantitatively differs between virgin and mated *Harmonia axyridis* females, regardless of the color morph. Second, we hypothesized that males discriminate virgin and mated females, preferring copulating with virgin females, rather than previously mated ones, to avoid sperm competition and subsequently

increase their fitness. CHCs were solvent-extracted before being quantified and identified by gas chromatography. We found no qualitative differences between mated and unmated females irrespective of the morph; however, quantitative differences were detected. Specifically, the CHC profiles of mated females presented higher concentrations of alkenes, including 9-pentacosene, 9-heptacosene, and 9-hentriacontene. During dual-choice behavioral assays, males equally copulated with virgin and mated females. Our results suggest that there is no CHC-based discrimination strategy in virgin males of *H. axyridis* between virgin and once-mated females. We discuss alternative strategies that might be used in this lady beetle species.

**Keywords:** Multicolored Asian lady beetle, sexual competition, mate selection, chemical signal, CHC, mating history, multiple mating

## 1 Introduction

The males of both vertebrates and invertebrates in many species are able to discriminate between virgin and mated females using chemical cues (Thomas 2011). Although some studies report no preference or a preference for mated females (Wenninger et al. 2008), males tend to prefer unmated females because they provide them with a higher fitness level. This phenomenon is particularly true for polyandrous insect species where females are generally less responsive to males after several mating events (Osawa 1994, de Jong et al. 1998). Among the observed strategies used to limit intraspecific competition, some males have adapted their copulatory behavior to remove previous sperm loads or allocate sperm strategically (in terms of quantity and/or quality) depending on the intensity of sperm competition (Birkhead & Hunter 1990, Thomas & Simmons 2007, Chaudhary et al. 2016).

Cuticular hydrocarbons (CHCs) are free lipids and the major components of the outer waxy layer of an insect cuticle (Lockey 1988). They are mostly composed of linear n-alkanes, linear alkenes and ramified saturated. CHCs are often species- and gender-specific (Pattanayak et al. 2014) and may provide information about the age (Cuvillier-Hot et al. 2001, Everaerts et al. 2010) as well as the sexual maturity of sexual partners (Dillwith et al. 1983). In some species of insects, males detect the presence and estimate the relative abundance of female CHCs to evaluate the presence of competitors as well as the mating status of the female. Such a strategy is used by *Teleogryllus oceanicus* Le Guillou (Orthoptera: Gryllidae), whose males adjust the quality of their ejaculate when detecting the presence of specific CHC (Thomas & Simmons 2009). CHCs are also used to discriminate between mated and virgin females (Carazo et al. 2004, Thomas & Simmons 2009, Thomas 2011): The males of *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) detect previously mated females due to CHCs and, consequently, increase the duration of copulation (Friberg 2006). Moreover, the cuticular hydrocarbon profile informs individuals about the female receptivity to mating (Fassotte et al. 2016) or sexual history (Howard & Blomquist 2005). Hydrocarbons are commonly transferred between mating partners as a strategy for sexual competition purposes (Thomas 2011, Ingleby 2015). CHCs hence appear to be crucial in insect chemical communication and mate selection.

Like in many animal species, copulation in coccinellids is the result from a complex multicomponent signaling (e.g. Bro-Jørgensen 2010). In coccinellids, males are first attracted at a distance by the female sex pheromone (Fassotte et al. 2014). Then, a pre-copulatory mating phase, including four characteristic steps ('watch at a distance', 'examine', 'mount' and 'attempt to copulate') is typically observed, and allows a male to make the choice of mating or leaving a female (Osawa 1994). *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) is a strongly invasive and highly polyphagous ladybird beetle (Koch 2003, Soares et al. 2008). Within its native range (China), this species presents a wide range of elytral color patterns that can be divided into two general phenotypes on the basis of background coloration: melanic (dark) and succinic/non-melanic (light) (Ueno et al. 1998). Such variability in colour morph has been shown to affect the male mating success (Ueno et al. 1998). The courtship behavior of males involves a latent period before copulation during which the male gets close to the female, examines her at a distance, mounts and attempts to copulate (Obata 1987). After genital connection, *H. axyridis* males display a series of stereotyped bouts of abdominal shaking, interspersed with resting intervals, which allow sperm transfer (Obata 1987). The copulation typically lasts 2 to 3 h (Wang et al. 2009, Nedved & Honek 2012). Chemical, visual and tactile cues are potentially involved in courtship behavior of this species, and allow the male to evaluate the ovarian development of a female, for instance (Osawa 1994). Several studies suggest that the chemical signals released by female coccinellids or present on their elytra have a determinant role in the courtship process (e.g., Obata 1987, Hemptinne et al. 1996, Omkar & Srivastava 2002, Omkar & Pervez 2005). However the relative importance of qualitative and quantitative profile of cuticular hydrocarbons (CHCs) in coccinellid mate recognition is still a subject of debate. To address this knowledge gap, we analyzed the CHC qualitative and quantitative profile of virgin and mated females of the Asian lady beetle *H. axyridis*, taking the color morph into account (Awad et al. 2017). Furthermore, we tested whether males discriminate among females according to their mating status during laboratory assays. We hypothesised that (i) the CHC profile differ between virgin and mated females, and (ii) males discriminate between virgin and mated females to overcome sperm competition and subsequently increase their fitness.

## 2 Materials and methods

### 2.1 Biological material

Melanic and non-melanic adult *H. axyridis* were collected in the vicinity of Beijing (China) in 2014 (Latitude: 39.9, Longitude: 116.2). The insects were placed together in an aerated plastic box (36 × 15 × 8 cm), and were fed with sugar lumps, a water-impregnated sponge, and bee-collected multifloral pollen. Pea aphids (*Acyrtosiphon pisum* Harris) were added ad libitum to stimulate mating and oviposition. Corrugated filter paper was used as egg laying material, and was changed when eggs were deposited. The eggs were placed in another plastic box to avoid cannibalism, and the larvae

were fed ad libitum with pea aphids every day until pupation. Three days after emergence, lady beetles acquire their final pigmentation, and the gender may be determined using morphological characteristics, as described by McCornack et al. (2007). Males and females were then kept in separate containers until the bioassays. All insects were reared in controlled environment chambers, with a 16 h-light photoperiod, at  $24 \pm 1$  °C, and  $45 \pm 15\%$  relative humidity (RH). Because the profiles of cuticular compounds are sometimes age-specific (Everaerts et al. 2010), we only tested lady beetles of the same age, and all originated from the first generation obtained in the laboratory following insect collection. The experiments were conducted on approximately 30 days old sexually mature adults.

## 2.2 Cuticular profiles

Four days before CHC extraction, aphids were added ad libitum every day to the rearing boxes. Couples were constituted by picking one female and one male and were placed in separate Petri dishes (diameter: 5.5 cm, height: 1.5 cm). If mating was observed, the female was placed at  $-80$  °C for 5 min. Because the male only touches the posterior area of female elytra during the body shaking stage (Obata 1987), we cut off the posterior half part of female elytra, which were then directly immersed in 200  $\mu$ l of n-hexane (95% purity, Sigma-Aldrich, St. Louis, MO, USA) for 7 min under constant agitation. This process was repeated for ten mated females from each color morph (i.e., melanic morph *spectabilis* and non-melanic morph *axyridis*). The elytra cut from ten virgin females from each color morph were also included in the assay as controls. The samples were kept at  $-18$  °C until gas chromatography analysis.

For quantification purposes, 1  $\mu$ l of each sample were injected on a gas chromatograph (Thermo Trace 1300) coupled with an ELITE 5HT (PerkinElmer) column (15 m  $\times$  0.25 mm  $\times$  0.1  $\mu$ m) and a flame ionization detector set at 330 °C. The injector (AS 1310) was set at 300 °C and was on a splitless mode. The carrier gas was helium (1.50 ml/min). The programmed temperature was 40 °C for 5 min, followed by a gradual increase of 10 °C/min to 330 °C, which was held for 10 min. The limit of detection (LOD) was assumed to be reached when the peak height of the component was less than or equal to three times the ratio of the signal height on the noise height. The limit of quantification (LOQ) was assumed to be two times higher than the LOD. We quantified each saturated compound according to a calibration curve based on the solutions of their analytical standards (Alkanes Mix 10, Ehrenstorfer), which were injected at increasing concentrations (1, 5, 10, and 25  $\mu$ g/ml). Unsaturated compounds were quantified based on the calibration of the corresponding alkane. Because cuticular hydrocarbons were present at low concentrations, the samples were pooled according to the mating state and morph type for identification purposes. Then, each pooled extract (n = 4) was concentrated (at  $\pm$  200  $\mu$ g/ml) under a gentle stream of nitrogen.

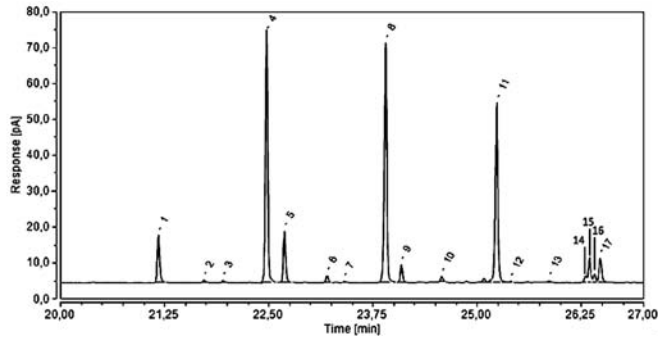
CHC identification was performed on a gas chromatograph equipped with a HP-5 column (30 m  $\times$  0.25 mm  $\times$  0.25  $\mu$ m) coupled with a mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The injector was set at 300 °C and injections were made in splitless mode using 1  $\mu$ l of each sample. The carrier gas was helium

(1.5 ml/min). The initial temperature was held at 40 °C for 2 min, followed by a gradual increase of 10 °C/min, with a final hold of 10 min at 320 °C. The mass spectra were recorded in the electron impact mode at 70 eV (source temperature of 230 °C, scanned mass range: 30 to 600 m/z). Saturated compounds were identified by their characteristic molecular ion, and the injection of pure n-alkane standards (Alkanes calibration standard from nC8 to nC40, purchased from Sigma-Aldrich, St. Louis, MO, USA). To localize the double bound monounsaturated alkenes, the molecule of interest have been submitted to epoxydation (Mallet et al. 1985). Epoxydation was performed on 400µl extract, the n-hexane of which was evaporated under a gentle stream of nitrogen. Then, 40µl chloroform (99.5% purity, Sigma-Aldrich, St. Louis, MO, USA) was added, followed by 40 µl chloroformic solution of m-chloroperbenzoic acid (25 mg/ml). The resulting blend was gently agitated for 2-h. Then, 400 µl of an aqueous solution containing sodium bisulfite (50 mg/ml) and sodium bicarbonate (50 mg/ml) was added, followed by 200 µl chloroform. With a syringe, we extracted the chloroformic phase, and the chloroform was evaporated under a gentle stream of nitrogen. Finally, the hydrocarbons were solubilized in 25 µl of n-hexane, and the resulting sample was stored at -18 °C until GC-MS analysis.

The concentrations of CHCs (i.e., absolute abundances, ng/individual) were compared among color morphs and mating status using a two-ways analysis of variance (two-ways ANOVA) on rank-transformed data as normality and homoscedasticity were not met nor restored with usual data transformation (Zimmerman & Zumbo 1993). Differences in CHCs profiles (i.e., relative abundances) among morphs and mating status were then assessed using perMANOVA (Bray-Curtis dissimilarity index, 999 permutations, “adonis” command) and multiple pairwise comparisons with Bonferroni’s adjustment after testing for multivariate homogeneity (“betadis-per” command) (R-package vegan, Oksanen et al. 2016). Differences were visually assessed on a non-metric multidimensional scaling (nMDS) ordination using a Bray-Curtis similarity matrix, three dimensions and 50 runs. A stress value is given to calculate how well the particular configuration produces the observed distance matrix (conventional cutoff of < 0.2). Indicator compound analyses on absolute abundances were also performed to identify CHCs that were indicative of a particular color morph and/or mating state (“indval” command) (R-package labdsv, Roberts 2016). All univariate and multivariate statistics were conducted in R version 3.3.1 (R Development Core Team 2016) using the 7 CHCs that were above the LOQ.

### 2.3 Bioassays

After 4-days of stimulation with aphids, females and males of the same morph were placed in couples in a Petri dish (5 cm diameter) until the male has finished shaking his body against the female. At this point, the mated female was placed in a new Petri dish with a virgin female and a virgin male. The trio was observed to identify the first female being mounted by the virgin male. The proportion of males initiating mating was recorded, as well as the latent period before copulation. The frequency with which a male preferred mating with a virgin female was analyzed by a two-sided bino-



**Fig. 1.** Gas chromatogram of the CHC profile emitted by an *H. axyridis* female. The components were identified by GC-MS analysis as (1) C23, (2) 9C24:1, (3) C24, (4) 9C25:1, (5) C25, (6) 9C26:1, (7) C26, (8) 9C27:1, (9) C27, (10) 9C28:1, (11) 9C29:1, (12) C29, (13) 9C30:1, (14) C31:2, (15) C31:2, (16) C31:2, (17) 9C31:1.

mial test based on the null hypothesis that the probability of partner choice is equal to 50%. This analysis was conducted for the melanic couples, the non-melanic couples, and both sets of data pooled together, for a total of 26 trios (13 trios per morph).

### 3 Results

#### 3.1 Do the CHC profiles of virgin and mated *H. axyridis* females differ?

All the *H. axyridis* females, whatever the color morph and the mating experience, display the same 17 cuticular components: six alkanes, three polyunsaturated alkenes and eight monounsaturated alkenes, with 9-heptacosene being the most abundant in all females (Fig. 1). Among them, seven cuticular chemicals were sufficiently abundant to allow their quantification (>LOQ).

According to the two-way ANOVA, the total quantity of CHCs is significantly higher in mated females than in virgin ones ( $F_{1,36} = 12.657$ ,  $p = 0.001$ ). This is mainly due to the higher amounts of n-tricosane, 9-pentacosene, 9-heptacosene, and 9-hentriacontene in mated females (Table 1, Table 2). Although there is no significant difference between melanic and non-melanic females with regards to the total CHCs content ( $F_{1,36} = 1.431$ ,  $p = 0.239$ ), non-melanic females display a higher abundance of 9-nonacosene compared to melanic ones (Table 1, Table 2).

The cuticular CHC profile (i.e., relative abundances) is not significantly different between mated and virgin females ( $F_{1,38} = 1.470$ ,  $p = 0.235$ ) although some compounds are associated to a small extent (i.e., low indicator value) with the mated

**Table 1.** Identification and quantification of the cuticular hydrocarbons collected from *H. axyridis* elytra based on the female mating status and color morph.

Peak numbers	Name	CHCb	R <sup>f</sup> c	R <sup>id</sup>	M <sup>+e</sup>	Quantity (ng/ladybeetle) ( $\pm$ SD)					
						Melanic			Non-melanic		
						Mated	Virgin	Mated	Virgin	Mated	Virgin
1	n-Tricosane	C23	21.173	2300	324	403 $\pm$ 216	373 $\pm$ 335	339 $\pm$ 49	233 $\pm$ 65		
2	9-Tetracosene	9C24:1	21.717	2375	336	<LOQ	<LOQ	<LOQ	<LOQ		
3	n-Tetracosene	C24	21.947	2400	338	<LOQ	<LOQ	<LOQ	<LOQ		
4	9-Pentacosene	9C25:1	22.471	2475	350	1,264 $\pm$ 668	743 $\pm$ 244	1,014 $\pm$ 413	711 $\pm$ 276		
5	n-Pentacosane	C25	22.687	2500	352	348 $\pm$ 132	382 $\pm$ 249	357 $\pm$ 95	301 $\pm$ 79		
6	9-Hexacosene	9C26:1	23.197	2575	364	<LOQ	<LOQ	<LOQ	<LOQ		
7	n-Hexacosane	C26	23.402	2600	366	<LOQ	<LOQ	<LOQ	<LOQ		
8	9-Heptacosene	9C27:1	23.901	2675	378	1,757 $\pm$ 671	1,176 $\pm$ 238	1,721 $\pm$ 313	1,312 $\pm$ 267		
9	n-Heptacosane	C27	24.087	2700	380	224 $\pm$ 78	328 $\pm$ 300	233 $\pm$ 91	176 $\pm$ 26		
10	9-Octacosene	9C28:1	24.573	2775	394	<LOQ	<LOQ	<LOQ	<LOQ		
11	9-Nonacosene	9C29:1	25.235	2875	406	790 $\pm$ 288	635 $\pm$ 229	1,191 $\pm$ 429	1,085 $\pm$ 472		
12	n-Nonacosane	C29	25.4	2900	408	<LOQ	<LOQ	<LOQ	<LOQ		
13	9-Triacontene	9C30:1	25.863	2975	420	<LOQ	<LOQ	<LOQ	<LOQ		
14	Hentriacontadiene	C31:2	26.302	3045	432	<LOQ	<LOQ	<LOQ	<LOQ		
15	Hentriacontadiene	C31:2	26.347	3055	432	<LOQ	<LOQ	<LOQ	<LOQ		
16	Hentriacontadiene	C31:2	26.408	3065	432	<LOQ	<LOQ	<LOQ	<LOQ		
17	9-Hentriacontene	9C31:1	26.473	3075	434	309 $\pm$ 84	202 $\pm$ 41	351 $\pm$ 96	245 $\pm$ 60		
Total						5,096 $\pm$ 1,884	3,840 $\pm$ 1,108	5,207 $\pm$ 807	4,064 $\pm$ 696		

LOQ Limit of quantification

a Correspondence with the peak identified on the gas chromatogram (Fig. 1)

b Abbreviation of hydrocarbon name

c Retention time

d Retention index

e Molecular ion



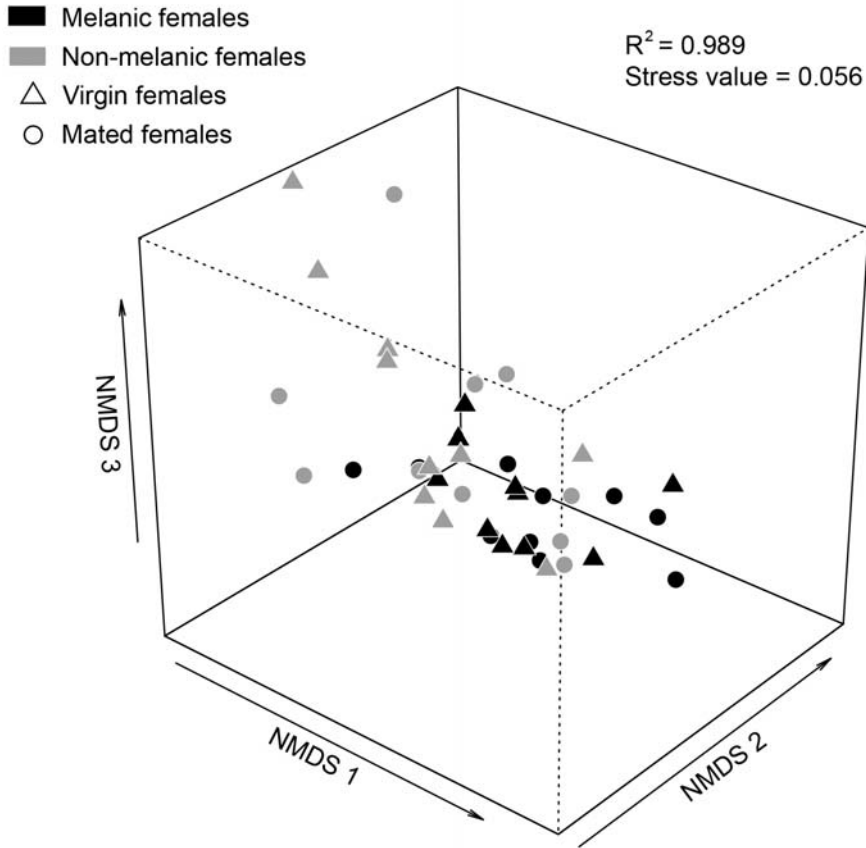
**Table 2.** Two-ANOVA on the absolute quantity of total CHC content and the individual compounds (> LOD, ng/ladybeetle) according to mating status, color morph and their interaction.

Name	Factor		
	Colour morph	Mating status	Colour morph: Mating status
n-Tricosane	F = 0.203	F = 10.720	F = 1.169
	p = 0.6550	p = 0.0023**	p = 0.287
9-Pentacosene	F = 0.333	F = 7.992	F = 0.030
	p = 0.568	p = 0.008**	p = 0.863
n-Pentacosane	F = 0.045	F = 1.362	F = 0.101
	p = 0.833	p = 0.251	p = 0.752
9-Heptacosene	F = 1.558	F = 19.962	F = 0.004
	p = 0.220	p < 0.001***	p = 0.948
n-Heptacosane	F = 1.003	F = 0.950	F = 1.115
	p = 0.323	p = 0.336	p = 0.298
9-Nonacosene	F = 15.544	F = 2.139	F = 0.198
	p < 0.001***	p = 0.152	p = 0.659
9-Hentriacontene	F = 2.985	F = 21.858	F = 0.332
	p = 0.093	p < 0.001***	p = 0.568
Total content	F = 1.431	F = 12.657	F = 0.046
	p = 0.239	p = 0.001**	p = 0.831

females, namely 9-pentacosene (indicator value = 0.61,  $p = 0.015$ ), 9-hentriacontene (indicator value = 0.60,  $p = 0.012$ ) and 9-nonacosene (indicator value = 0.58,  $p = 0.007$ ). By contrast the CHC profile is significantly different depending on the color morph ( $F_{1,38} = 5.583$ ,  $p = 0.004$ ), with 9-nonacosene being indicative of non-melanic females but to a small extent (indicator value = 0.62,  $p = 0.021$ ). The analysis of interaction (i.e., color morph:mating state) shows that this difference between color morphs is mainly due to virgin females that differ significantly between the color morphs ( $p = 0.011$ ) as well as from mated females of the other color morph (melanic virgin vs. non-melanic mated females,  $p = 0.031$ , non-melanic virgin vs. melanic mated females,  $p = 0.017$ ), while CHC profile is not significantly different between mated melanic and non-melanic females ( $p = 0.059$ ) (Fig. 2).

### 3.2 Do *H. axyridis* virgin males prefer to copulate with virgin or mated females?

Males initiated copulation within the first 10 minutes of observation in all conducted bioassays. For the melanic morph, as well as for the non-melanic morphs, we found



**Fig. 2.** NMDS ordination plots based on Bray-Curtis dissimilarity index calculated on relative abundances of CHCs from the posterior half of a female elytra pair showing 3D configuration.

that the probability of a male choosing a mated female in each morphological group was close to 50% ( $p > 0.999$ ,  $n = 13$  for each morph). The same result is observed after pooling both data sets ( $p = 0.422$ ,  $n = 26$ ). The latent period preceding copulation was similar between morphs ( $t$ -value =  $-0.04$ ,  $p = 0.970$ ) and is not influenced by the mating status of the selected female ( $t$ -value =  $0.68$ ,  $p = 0.502$ ).

#### 4 Discussion

In *Harmonia axyridis*, males exhibit a characteristic behavior before copulation that includes five characteristic steps (e.g. Obata 1987, Omkar & Srivastava 2002, Fassotte et al. 2016): ‘approach a female’, ‘watch at a distance’, ‘examine’, ‘mount’

and ‘attempt to copulate’. Such as many other animal species, mate location and decision of mating is taken according to a complex multicomponent signaling, involving chemical, visual, tactile and other behavioral cues (Hodek & Ceryngier 2000, Bro-Jørgensen 2010). While volatile pheromones are involved during the first step (Fassotte et al. 2014), visual inspection of the potential mate occurs in the following one, with colour of the elytra as possible cue for mate choice (Obata 1987, Hodek & Ceryngier 2000). Contact is made during the third step, with the male placing its antennae and forelegs on the female body surface, allowing body size and shape identification. Finally, the male mounts the female and tries to copulate by bending the abdomen downwards (Obata 1987). If volatile and non-volatile chemical stimuli are important, they probably overlap with other stimuli that can be useful to assess the mating status of an insect (e.g. Romano et al. 2016, 2018). However, the relative importance of each of these cues in the sexual behavior of this lady beetle species remains to be clarified.

One mating event is obviously sufficient to induce change in cuticular hydrocarbons on the elytra of *H. axyridis* females. Quantification analyses highlight a significant increase in total CHC concentration following copulation. This increase is mostly due to three specific monounsaturated alkenes (i.e., 9-pentacosene, 9-heptacosene, and 9-hentriacontene) that are emphasized as indicative compounds of the mated status (i.e., indicator compound analysis). Such change in CHCs might arise from deposition by the male partner with the transfer of hydrocarbons between the sexual partners being a common strategy in sexual competition (Ingleby 2015). Such behavior has already been shown in *D. melanogaster* with the mature males marking mated females with 7-tricosene, which acts as anti-aphrodisiac to other competitors (Scott 1986). A similar strategy of repelling competitors has been also highlighted in pierid butterflies, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) and Heliconius butterflies (Happ 1969, Gilbert 1976, Andersson et al. 2003). Post-mating modifications in female CHC profile could also increase the male fitness if they are able to discriminate virgin females. Relying on CHC profile is the most common mechanism used by insect males to increase their fitness though favoring virgin females (Carazo et al. 2004). However, according to our bioassays, *H. axyridis* males do not use modifications in CHC profiles to avoid copulating with mated females (i.e., no preference in their first mate choice between a virgin and a mated female). It is yet possible that males adapt their quantity and/or quality of sperm based on female CHC differences, which remains to be tested. As we have recorded changes in CHC composition only after a single mating event, we might assume that a greater number of mating events could imply stronger chemical modifications, leading then to a higher chance of discrimination. If this hypothesis is confirmed, then males would be able to consider the competition level (i.e., the intensity of chemical marking) rather than the dual status “mated versus virgin”. Caution has also to be paid as we only considered virgin males in our bioassays. Mated males could display a different ability in female selection, being more selective than virgin ones based on CHC cues.

As polyandry is often observed in coccinellids on one hand, and as the first male of *H. axyridis* copulating with a particular female has higher proportion of offspring on the other hand (Laugier et al. 2013), males could be able to discriminate females

based on other cues (Johansson & Jones 2007). Other life history parameters, such as female age could be evaluated as a reliable indication to the risk of sperm competition. This suggestion was not considered in our experimental design since all tested females were of the same age. However, in some insect species, the age of the female is an important criterion for mate choice as it might be correlated with the probability of previous mating events. Unfortunately, only a few studies have documented the impact of female age on mate discrimination (Thomas 2011). Moreover, the effect of multiple mating on females is often debated, and not only in insects (Arnqvist & Nilsson 2000). In *H. axyridis*, multiple inseminations are common (Ueno 1996) but lead to severe costs for females, such as energy loss, food deprivation, reduced longevity, oviposition, and infections risks (Daly 1978, Osawa 1994, Omkar & Mishra 2005, Omkar & Sahu 2012). Alternatively, egg production, fertility as well as offspring performance might be enhanced, particularly in species where sperm mixing occurs (Thomas 2011). This latter trend has been observed in several coccinellid species, such as *Propylea dissecta* Mulsant (Omkar & Pervez 2005), *Coelophora saucia* Mulsant (Omkar et al 2010), *Anegleis cardoni* Weise (Omkar & Sahu 2012), *Coccinella septempunctata* L. (Srivastava & Omkar 2005) and *Adalia bipunctata* L. (Haddrill et al. 2007). For species where females benefit from multiple mating (i.e., increasing their reproductive success), males would be less likely to exclude previously mated females and prevent multiple mating. Instead of rejecting a mated female, males of a polyandrous species should preferentially allocate their ejaculate strategically, by modulating sperm quality and/or quantity (Thomas & Simmons 2007, 2009) or by evacuating the sperm of previous males (Birkhead & Hunter 1990). Modifying their copulation behavior by copulating with mated females for longer might also increase the chances of males gaining paternity (Friberg 2006, Chaudhary et al. 2016).

Based on our bioassays, there is no discrimination strategy in virgin males of *H. axyridis* between virgin and once-mated females. However, this study brings new clues for further investigations. CHCs might be used to assess the competition level, with mated males being more efficient than virgin one to perform this CHC identification. Moreover, alternative strategies should also be considered. Multiple signaling (involving a composite signal that comprises several components, each with signal properties) is common when it comes to animal reproductive behavior (Bro-Jørgensen 2010, Romano et al. 2017), and is likely to occur in this lady beetle species, which has overcome fluctuating ecological and social environments, especially during its invasion process.

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