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ORIGINAL CONTRIBUTION

Is conspecific substrate marking a long-term external memory of previously colonized overwintering sites in *Harmonia axyridis*?

D. Durieux[1], B. Fassotte[1], M. Vanderplanck[2], Y. Brostaux[3], C. Fischer[4], G. Lognay[4], E. Haubruge[1] & F. J. Verheggen[1]

1 Functional and Evolutionary Entomology, Gembloux Agro-Bio Tech, University of Liege, Gembloux, Belgium
2 Laboratory of Zoology, Research Institute of Biosciences, University of Mons, Mons, Belgium
3 Applied Statistics, Computer Science and Mathematics, Gembloux Agro-Bio Tech, University of Liege, Gembloux, Belgium
4 Laboratory of Analytical Chemistry, Analysis Quality and Risk, Gembloux Agro-Bio Tech, University of Liege, Gembloux, Belgium

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Correspondence
Delphine Durieux (corresponding author), Functional and Evolutionary Entomology, Gembloux Agro-Bio Tech, University of Liege, Passage des Déportés 2, 5030 Gembloux, Belgium.
E-mail: entomologie.gembloux@ulg.ac.be

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Abstract

The multicoloured Asian ladybeetle, *Harmonia axyridis* (Pallas), aggregates inside dwellings during winters to survive the cold. This beetle uses chemical cues coming from congeners to select an overwintering site. Recent research has shown that they preferentially gather at places where conspecifics previously laid a substrate marking made up of saturated and unsaturated hydrocarbons. Some authors have reported that *H. axyridis* colonizes the same overwintering sites from 1 year to another. Herein, the hypothesis that this substrate marking is used by *H. axyridis* to settle in the same aggregation sites from one winter to another was tested. To this aim, the temporal modification in the chemical profile of the hydrocarbon marking was studied by performing chromatographic analyses. After 1 year, the overall profile was modified qualitatively and quantitatively: the unsaturated hydrocarbons were no longer detected while some saturated hydrocarbons were still present in large quantities. In a behavioural assay conducted in the laboratory, the 12-month-old marking did not induce the aggregation of *H. axyridis*. This result indicates that the chemical markings left by conspecifics during a previous aggregation period in an overwintering site are not sufficient to induce the gathering of the newly arriving individuals.

Introduction

Various insect groups, including ants (Depickère et al. 2004), termites (Heidecker and Leuthold 1984), cockroaches (Rivault and Cloarec 1998), honeybees and wasps (Butler et al. 1969), use chemical cues left by congeners on the substrate, i.e., substrate markings, to assess their environment. Two kinds of substrate markings can be distinguished: territorial markings and home-range markings (Hölldobler and Wilson 1990). The first form serves to identify the area which is considered as belonging to a group of individuals (Hölldobler and Wilson 1990). The group members will then defend it against intruders. This defence does not necessarily imply the killing of the foreign insects, but simply their expulsion from the guarded area (Devigne and Detrain 2002). In contrast, the second form of marking indicates the suitability of an area and involves no defence against intruders (Hölldobler and Wilson 1990). In this case, these substances can be used to locate a nest, a resting site or foraging site (Rivault and Cloarec 1998; Devigne et al. 2004; Lenoir et al. 2009). Such home-range marking does not necessarily result from an active deposition. These cues can be formed with various materials laid passively on the substrate. For instance, the passive deposition of cuticular hydrocarbons is used as home-range marking by the German yellowjacket, *Vespula germanica* L., and the ant *Lasius niger* L., respectively to locate the nest entrance and assess the distance of a foraging site from the nest (Steinmetz et al. 2003; Jandt et al. 2005; Devigne and Detrain 2006).

In the Coccinellidae, the use of such external cues is well documented. Several studies have confirmed the participation of larval footprints in deterring females from ovipositing in a place where conspecifics are already present (Hemptinne et al. 2001). More recently, it has been demonstrated that the invasive alien multicoloured Asian ladybeetles, *Harmonia axyridis* (Pallas), use substrate marking in the selection of their overwintering sites (Durieux et al. 2012). A marking composed of saturated and unsaturated long-chain hydrocarbons was collected inside these resting sites and clearly induced the retention of overwintering individuals in a laboratory bioassay. Evidence suggests that the chemical marking ensured the cohesion of the cluster formed by the beetles during winter.

Harmonia axyridis is a species native to Southeast Asia. It has been introduced into North America and Europe in the 20th century to biologically control aphid and coccid populations (Brown et al. 2011). Following these introductions, this exotic species is now well established on these continents (Brown et al. 2011), surviving cold winters by aggregating inside houses and buildings (Labrie et al. 2008). This adaptive behaviour causes annoyance as a result of the large number of ladybeetles which can be found inside dwellings, as well as by the potential induction of some allergic reactions in the inhabitants (Sloggett et al. 2011). According to Obata (1986), overwintering sites colonized by this pest remain stable for years. In this study, we have investigated whether chemical substrate markings are used by *H. axyridis* to colonize the same aggregation sites from one winter to another. In that order, the temporal modification in the chemical profile of the hydrocarbon mark was studied using gas chromatography–mass spectrometry analyses. The biological activity of the marking aged for 1 year was also tested to assess its retention potential.

Material and Methods

Biological material

One thousand multicoloured Asian ladybeetles were collected from infested dwellings located in the region of Gembloux (Belgium) during December 2010. Thereafter, 50 other individuals, intended to be used in the bioassays, were collected in the same conditions during December 2011. All these adults were placed in 36 × 15 × 8 cm aerated plastic boxes for 1 week at most until the beginning of the test. To maintain them under aggregation conditions, they were kept in the dark, at 15 ± 1°C without any food or water. This temperature was in accordance with the mean temperature recorded in the investigated aggregation sites (14.37 ± 3.66°C, n = 10).

Collection of the aggregation chemical marking

The experimental device consisted of a glass Petri dish (diameter: 18 cm, height: 4 cm), in which the inside walls were covered with a strip of brass wire mesh (90 × 90 mesh – Innopress Sarl, Touvois, France) that had been previously cleaned with norvanol (ether-denatured ethanol: VWR International, Haasrode, Belgium). Such a device was used further to previous observations showing that *H. axyridis* preferentially aggregates vertically and especially on this substrate (D. Durieux, B. Fassotte, M. Vanderplanck, Y. Brostaux, C. Fischer, G. Lognay, E. Haubruge, F.J. Verheggen, personal observations). Two hundred ladybeetles collected during December 2010 (males and females not being differentiated) were introduced inside the arena and were left for 24 h. The Petri dish had been previously cleaned with the liquid detergent RBS T 105 (Chemical Products R. Borghgraef, Brussels, Belgium) and norvanol. The arena was placed in the dark at 15 ± 1°C in the hope that the ladybeetles would cluster on the strip. After that time, insects were removed and the strip area where they had aggregated was collected and divided into twelve pieces with similar surface areas. Five replicates were carried out.

Brass strip pieces were then kept at room temperature (20.28 ± 7.24°C, mean ± SE calculated over 1 year) on an inner window sill situated above a radiator. Such an environment was similar to the one often colonized by overwintering *H. axyridis* in dwellings (D. Durieux, B. Fassotte, M. Vanderplanck, Y. Brostaux, C. Fischer, G. Lognay, E. Haubruge, F.J. Verheggen, personal observations). Moreover, the selected window was directed to the south, which is the preferred direction of *H. axyridis* to aggregate (Kidd et al. 1995; Raak-van den Berg et al. 2012). Our observations in infested dwellings support this statement as the multicoloured Asian ladybeetles aggregate facing the south in 37% of cases (instead of 12.5%, both main and intermediate directions being taken into account).

The chemical compounds of the fresh marking, i.e., the cue having contributed to the formation of the overwintering ladybeetles aggregation, were identified, and this cue was characterized in terms of compound ratios. In that order, one strip piece of each replicate was directly placed in 1.25 ml of n-hexane (HPLC grade, 95% purity – Fisher Scientific, Loughborough, Leicestershire, UK) to extract all of the compounds. Thereafter, the chemical identification and the determination of compound ratios were repeated

after 1, 2, 3, 6, 9 and 12 months. After each of these stretches of time, one strip piece of each replicate was removed from the window sill and the same extraction operation as described above was performed. All extracts were then used for chemical analyses.

The remaining five strip pieces of each replicate were left in the experimental conditions (i.e., on the window sill) for 1 year. After that time, they were used in a bioassay to assess the retention capacity of this cue 1 year after being laid.

Temporal modification in the aggregation chemical marking profile

The extracted compounds were identified by gas chromatography–mass spectrometry (GC-MS). This analysis was performed by injecting 1 μl of the n-hexane extract onto an Agilent Technologies 6890N Network GC System equipped with a HP-5 (5% phenyl/95% methylsiloxane) column (30 m × 0.25 mm I.D.; film thickness 0.25 μm) coupled to an Agilent 5973 Network Mass Selective Detector (Agilent Technologies, Diegem, Belgium). The operating conditions were: a split ratio of 20 : 1, injector at 300°C; carrier gas: helium at 1.7 ml/min; temperature programme: from 40°C (held for 2 min) to 320°C at 10°C/min with a final hold of 10 min at 320°C. The mass spectra results were recorded in the electron impact mode at 70 eV (source temperature at 230°C, transfer line at 320°C, scanned mass range: 40–500 amu). The detected peaks were identified by their retention data and their characteristic fragmentation patterns. The identification of saturated compounds was then confirmed by the injection of n-alkanes standard (from C_9 to C_{40}). To identify the double bond position of monounsaturated compounds, an epoxidation using m-perchlorobenzoic acid was performed (Durieux et al. 2012). The identification of the position of methyl in branched alkanes was determined from the occurrence of C_nH_{2n+1} and C_nH_{2n} fragments after α-cleavage at the branch positions (McCarthy et al. 1968).

Given that the cue was naturally laid by overwintering ladybeetles, the quantity of deposited marking on the strip portions used for these analyses was not uniform. To suppress any bias coming from these irregularities, the comparison between the extracts of different ages was carried out on the mean percentages of the substrate marking chemical compounds. These percentages were established using a Thermo Trace Fast GC (Thermo Electron Corporation, Interscience, Louvain-la-Neuve, Belgium) equipped with a flame ionization detector (FID at 310°C −300 Hz) and a Ph5 column (5 m × 0.1 mm × 0.1 μm). The initial temperature of the column was maintained at 40°C for 30 s and then was programmed to 310°C at 60°C/min with a final hold of 1 min at 310°C. The carrier gas was helium (0.5 ml/min) and the injection mode was 'split' (split ratio of 20 : 1) at 310°C.

Bioassay

The remaining strip pieces, aged 12 months, were pooled into five, with one piece coming from each replicate. The chemicals present on the five pieces were extracted in 3.75 ml of n-hexane. Each resulting mixture was deposited on 19 cm² of a brass strip (56.5 cm long, 3.5 cm high) that had been formerly cleaned with norvanol. The coated area surface was assessed as being the mean area surface of the five pooled strip pieces. Each metal strip was then placed sideways into a Petri dish that had been previously cleaned with RBS T 105 and norvanol.

Ten overwintering ladybeetles, collected in infested dwellings in December 2011, were released in the centre of the Petri dish, and their position was recorded every 10 min during the first hour and then after 1, 3, 5 and 8 h. Seven areas were defined inside the Petri dish (in an identical way to the one illustrated in Durieux et al. 2012) six identical portions (except for the presence of chemicals in one of them) and a 4-cm-diameter circle in the centre, defining a neutral area. With the available quantity of chemicals, five replicates could be performed. To avoid bias due to directional effects, the area containing the chemical cue was randomly oriented. The experiment was carried out in a growth chamber at 14 ± 1°C, in the dark and 55 ± 5% relative humidity. The suitability of this protocol to assess the retention potential of a marking on overwintering *H. axyridis* has already been demonstrated in a previous work (Durieux et al. 2012).

Statistical analyses

To detect significant temporal modification(s) in the chemical profile of the substrate marking over 1 year, permanova ('adonis' command, R-package vegan, Oksanen et al. 2010) was performed on the relative abundances of each hydrocarbon, using the Euclidean distances matrix and 999 permutations. Permanova is a permutation-based version of the multivariate analysis of variance (manova). Like conventional analyses of variance, permanova calculates an F statistic by taking the ratio of the between-group sum of squares and the within-group sum of squares. Prior to this permutational analysis of variance, the

multivariate homogeneity of within-group covariance matrices had been verified using the 'betadisper' function (vegan package, Oksanen et al. 2010) implementing Marti Anderson's testing method. When P-value returned by permanova was significant ($P < 0.05$), multiple pairwise comparisons were conducted on the data to detect precisely the differences in the chemical composition of the substrate marking during its ageing process. To visually assess the chemical evolution of the substrate marking over 1 year, a non-metric multidimensional scaling (NMDS) ordination was performed using a Euclidean distances matrix, two dimensions (applying a conventional cut-off of <0.2 for the stress value) and 50 runs to more fully explore the ordination space at that dimensionality. All multivariate data visualization and analyses were carried out in R version 2.9.1 (R Foundation for Statistical Computing, Vienna, Austria) using functions from vegan, ecodist (Goslee and Urban 2007), ellipse (based on standard deviation) (Murdoch and Chow 2007) and BiodiversityR (Kindt and Coe 2005).

Results of the bioassay were statistically analysed by adjusting a generalized linear mixed model (GLMMz) to the observed counts in the marked area vs. the unmarked ones. To take into account the non-independence of the different observation times, repetitions were introduced as a random effect, with a fixed time effect. A binomial error family was used for the model, with a logit link between the linear part of the model and the estimated probabilities. As only one of the six lateral areas was marked, the null hypothesis of no effect of the deposited chemical compounds was tested by comparing the observed probability of occurrence in the marked area against the theoretical value of one-sixth, corresponding to a random choice, using the coefficients of the adjusted GLMMz. The individuals located in the central area at each observation time were not included in the analysis. GLMMz analysis was performed with R statistical software v. 2.13.0. (R Foundation for Statistical Computing) and lme4 package v. 0.999375-40 (Bates et al. 2011).

To be able to draw a parallel between the bioassay results and the ones obtained in our previous study (Durieux et al. 2012), the ladybeetle percentage having participated has to be similar in the two behavioural experiments. To verify this fact, the proportion of individuals having made a choice in the assay presented herein was compared with the one previously recorded. Given that the observation 'having made a choice' produces binary data, a binary logistic regression (Minitab® 15.1.1.0, State College, Pennsylvania – $n = 30$, $\alpha = 5\%$) was performed, the function logit being used as link.

Results

Prior to our analyses, we ensured that the chemical profile of the present substrate markings freshly deposited by overwintering ladybeetles display the same cue as the ones highlighted in our previous study (Durieux et al. 2012), which are known for being responsible for the cohesion of the cluster (permanova: $F_{1,37} = 2.76$, $P = 0.086$).

Temporal modification of the aggregation chemical marking

The modifications in the chemical composition of the substrate marking during its ageing process are presented in table 1. Evidence is that marking chemical profile changes according to the time, which is statistically supported by the permanova result ($F_{6,28} = 22.87$, $P < 0.001$). The nMDS ordination arranged the chemical profiles of the substrate marking into two distinct groups according to NMDS 1: (i) the first one including months from 0 to 3 and (ii) the second one including months from 6 to 12. Whereas the group formed by the three last ageing months is clearly heterogeneous (all month extracts are mixed without significant difference; pairwise comparisons, $F_{1,8} = 0.11–0.67$, $P > 0.05$), the group composed of the first months (from 0 to 3) displays a gradient along NMDS 2 with significant differences detected between all the four extracts (pairwise comparisons, $F_{1,8} = 6.59–76.64$, $P < 0.01$) (fig. 1a). This month gradient highlights both a decrease in the percentage of unsaturated compounds and an increase in the saturated ones from month 0 to month 3 (fig. 1). From month 6, the unsaturated compounds were no longer detectable through our GC-MS analyses (table 1). Although the nMDS analysis displays no difference between extracts over 6 months of age, some chemical changes can be observed. The percentage of n-tricosane decreased, whereas the n-heptacosane, n-nonacosane and methyl-nonacosanes percentages increased from month 6 to 12. On the other hand, the levels of n-tetracosane and n-pentacosane remained stable. Moreover, it is important to notice that no additional peaks appeared with time.

Behavioural assay

The GLMMz analysis revealed a significant difference between the area containing the 12-month-old chemical marking and the five other areas only for the 10-min and 1-h time points (table 2). Moreover, the binary logistic regression shows no significant

Table 1 Modification in the substrate marking composition according to the time (mean percentage of each compound in the total hydrocarbon extract ± SE)

Chemical compound name	Abb*	Diagnostic ions (m/z)		Number of months after substrate marking was laid						
		Molecule	Epoxide	0	1	2	3	6	9	12
n-Tricosane	nC_{23}	324		18.29 ± 0.28	21.55 ± 0.73	32.35 ± 2.08	41.16 ± 1.87	23.81 ± 5.81	20.67 ± 7.40	17.30 ± 4.99
n-Tetracosane	nC_{24}	338		0.45 ± 0.02	0.66 ± 0.03	0.94 ± 0.12	1.07 ± 0.08	1.61 ± 0.47	3.01 ± 0.29	1.71 ± 0.70
9-Pentacosene	C_{25} :1	350	155–253	13.70 ± 0.30	13.42 ± 0.33	11.13 ± 0.89	7.63 ± 1.56	<LOD†	<LOD	<LOD
n-Pentacosane	nC_{25}	352		5.81 ± 0.09	7.76 ± 0.43	11.01 ± 0.72	14.51 ± 1.27	36.34 ± 1.70	36.80 ± 2.66	36.70 ± 0.93
Heptacosadiene	C_{27} :2	376		3.42 ± 0.15	2.81 ± 0.18	2.21 ± 0.19	2.55 ± 0.44	<LOD	<LOD	<LOD
9-Heptacosene	C_{27} :1	378	155–281	12.02 ± 0.23	11.98 ± 0.22	10.33 ± 0.78	7.00 ± 0.88	<LOD	<LOD	<LOD
n-Heptacosane	nC_{27}	380		1.68 ± 0.07	2.00 ± 0.16	3.51 ± 0.44	4.27 ± 0.73	14.33 ± 2.09	14.78 ± 1.76	17.46 ± 2.70
Nonacosadiene	C_{29} :2	404		21.54 ± 0.30	18.51 ± 0.77	10.52 ± 1.22	6.73 ± 1.12	<LOD	<LOD	<LOD
9-Nonacosene	C_{29} :1	406	155–309	5.39 ± 0.11	4.97 ± 0.13	3.98 ± 0.30	3.29 ± 0.31	<LOD	<LOD	<LOD
n-Nonacosane	nC_{29}	408		1.37 ± 0.08	1.65 ± 0.11	2.93 ± 0.36	3.82 ± 0.80	12.10 ± 1.35	12.55 ± 1.79	14.30 ± 2.43
13-&15-methyl -Nonacosanes	13-&15-Me-C_{29}	196–224–252–407		1.79 ± 0.08	2.01 ± 0.10	3.03 ± 0.36	3.36 ± 0.36	11.82 ± 1.40	12.19 ± 1.88	12.52 ± 1.34
Hentriacontadienes	C_{31} :2	432		12.98 ± 0.28	11.14 ± 0.43	6.90 ± 0.62	3.86 ± 1.10	<LOD	<LOD	<LOD
9-Hentriacontene	C_{31} :1	434	155–337	1.57 ± 0.06	1.57 ± 0.05	1.16 ± 0.12	0.74 ± 0.20	<LOD	<LOD	<LOD

*Abbreviation of hydrocarbon name.

†Limit of detection.

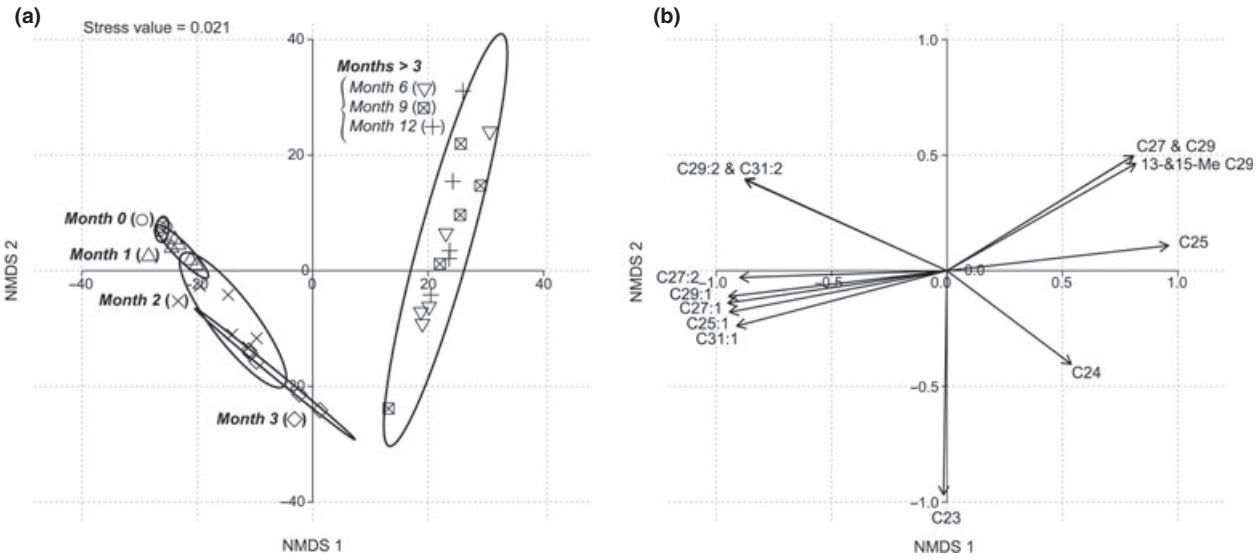

Fig. 1 nMDS ordination plot based on Euclidean distances calculated on relative abundances of chemical compounds in substrate marking collected inside aggregation sites through its ageing process over 1 year: (a) replicates within age showing the temporal modifications in chemical profile, and (b) chemical compound vectors. Stress value is indicated on the ordination.

Table 2 Mean percentage (±SE) of ladybeetles recorded in each of the six areas delimited in the bioassay testing the 12-month-old substrate marking at every observation time and associated results of the general linear model analyses comparing the distribution observed in the area containing the chemical compounds with those encountered in the five other areas

Time (min)	Area with marking	Control area 1	Control area 2	Control area 3	Control area 4	Control area 5	*Z* value	P value
10	38.00 ± 8.60	8.00 ± 5.83	6.00 ± 2.45	12.00 ± 5.83	18.00 ± 8.60	14.00 ± 2.45	2.44	0.015[a]
20	18.00 ± 5.83	10.00 ± 3.16	10.00 ± 3.16	28.00 ± 12.00	18.00 ± 4.90	14.00 ± 4.00	−0.13	0.898
30	28.00 ± 11.58	24.00 ± 5.10	8.00 ± 3.74	16.00 ± 9.27	12.00 ± 7.35	12.00 ± 4.90	1.06	0.288
40	24.00 ± 9.27	18.00 ± 5.83	8.00 ± 2.00	28.00 ± 8.60	14.00 ± 7.48	8.00 ± 2.00	0.57	0.566
50	32.00 ± 10.20	8.00 ± 2.00	10.00 ± 3.16	32.00 ± 12.41	10.00 ± 7.75	8.00 ± 3.74	1.53	0.126
60	38.00 ± 10.20	12.00 ± 5.83	8.00 ± 2.00	22.00 ± 12.00	2.00 ± 2.00	14.00 ± 2.45	2.35	0.019[a]
180	14.00 ± 7.48	10.00 ± 4.47	16.00 ± 9.27	34.00 ± 18.33	8.00 ± 3.74	18.00 ± 9.70	−0.78	0.434
300	22.00 ± 14.28	0.00 ± 0.00	10.00 ± 4.47	38.00 ± 18.55	14.00 ± 14.00	16.00 ± 11.66	0.32	0.749
480	26.00 ± 17.78	2.00 ± 2.00	12.00 ± 9.70	30.00 ± 20.00	10.00 ± 10.00	20.00 ± 12.65	0.82	0.411

[a]Statistical difference with $P < 0.05$.

difference in the percentage of individuals participating in the behavioural experiment between the previous study and the present one ($Z = -1.41$, $P = 0.159$).

Discussion

This study demonstrates that some hydrocarbons laid by *H. axyridis* in its aggregation sites are still present after 1 year. However, the chemical profile of the substrate marking deposited by overwintering beetles changes during ageing, with the greatest modifications being recorded during the first 3 months. The chemical analyses showed that the unsaturated compounds decreased according to time and were not quantifiable after 6 months. A diminution of saturated hydrocarbons also occurred but at a lower rate, with the increase in their percentage during the first 3 months only being as a result of the greater reduction of unsaturated chemicals (table 1). These changes in chemical profiles can be explained by the fact that, for the same carbon number, an alkene presents a greater vapour pressure, and so is more volatile, than the corresponding n-alkane (Jamart et al. 2009). Moreover, alkenes are less stable than alkanes; their decrease could then be accelerated by

oxidative degradation (Jamart et al. 2009). No additional compound was observed in chromatographic analyses, but it is probably linked to a great volatilization of the obtained degradation products explained by their lower molecular mass in comparison with the initial unsaturated molecules. Indeed, the fragmentation occurs at the more unstable position, which is, in this case, the double bond ones, located at the ninth carbon for mono-unsaturated hydrocarbons (Jamart et al. 2009). On the other hand, the evaporation rate of saturated compounds decreased with the increased carbon number. This is due to the decrease in the vapour pressure of an n-alkane with its molecular mass (Jamart et al. 2009).

It has recently been demonstrated, by performing the same behavioural assay with substrate markings freshly deposited by overwintering individuals in aggregation sites, that these cues have a retention potential on aggregating conspecific ladybeetles (Durieux et al. 2012). Although saturated hydrocarbons of such substrate marking partially persist during a period of 1 year, the remaining cue, presenting a new chemical profile, is not able to induce aggregation in newly arriving *H. axyridis* individuals. Indeed, the fact that very little preferences were shown by ladybeetles in favour of the marked area indicates that the marking does not have any retention capacity after 1 year. Given that the unsaturated hydrocarbons disappear more quickly, their contribution to the chemical profile modifications is greater than that of saturated compounds. Alkenes could therefore provide information to *H. axyridis* about the freshness of the substrate marking laid by previous conspecifics. On the other hand, the very weak preferences towards the aged substrate marking could also come from the decrease in the total hydrocarbon quantity, which might go below the detection threshold of ladybeetles. Indeed, it has been demonstrated in other insects, such as *Vespula germanica* (Fabricius) and *Apis mellifera* L., that such substrate marking need to reach a certain concentration to be perceived (Butler et al. 1969; Jandt et al. 2005).

As observed in *Lasius niger* (L.) (Depickère et al. 2004), the multicoloured Asian ladybeetles settle preferentially in a site previously marked by congeners (Durieux et al. 2012). It has been observed that *H. axyridis* colonizes the same overwintering sites from 1 year to another (Obata 1986). However, through these results, it appears that this behaviour is not due to the presence of a residual hydrocarbon cue in aggregation sites. Behaving differently according to the freshness of the substrate marking can benefit *H. axyridis*. Indeed, this will prevent beetles from colonizing a site previously marked if this one has changed and is no longer suitable. *H. axyridis* would then not be prisoner of a previous choice and trapped in suboptimal situations.

Some individuals of other ladybeetle species are often associated with *H. axyridis* clusters, such as *Adalia bipunctata* (L.) and *Oenopia conglobata* (L.) (D. Durieux, B. Fassotte, M. Vanderplanck, Y. Brostaux, C. Fischer, G. Lognay, E. Haubruge, F.J. Verheggen, personal observations). Therefore, it seems that the substrate marking studied in this work is more of a home-range marking than a territorial one. Ladybeetles do not feed nor reproduce during the overwintering period (Hodek 1973). It sounds logical that they have no interest in losing energy to defend this marked area. Moreover, the presence of a greater number of gathered individuals can be beneficial to *H. axyridis*, for instance by decreasing heat and water loss by individuals (Heinrich 1981; Dambach and Goehlen 1999). However, to date, the actual advantages given to *H. axyridis* by the formation of clusters remain unknown. Further studies are needed to understand the reasons that lead ladybeetles to aggregate during winter.

In conclusion, chemical markings left by conspecifics during a previous aggregative period in an overwintering site are not sufficient to induce the gathering at this place of the newly arriving individuals. The aggregative phenomenon generally results from the individual responses towards two types of cues: environmental heterogeneities and social interactions. In this case, the colonization of the same overwintering sites from 1 year to another might be due to the attraction of the first ladybeetles by some visual features. The importance of visual elements has already been demonstrated during the migratory flight (Obata 1986; Nalepa et al. 2005), and these could then be involved in the subsequent aggregation steps.

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