Is Contact Between Conspecifics Involved in the Cohesion of *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) Aggregations?

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Abstract The multicolored Asian ladybeetle, *Harmonia axyridis* (Pallas), exhibits a gregarious behavior during unfavorable winter conditions. Although this behavior is currently described as a phenomenon occurring only during winter, *H. axyridis* aggregations can also be observed outside overwintering conditions. However, the substrate markings previously highlighted as being involved in the wintry aggregation of this exotic species do not seem to be used by non-overwintering individuals to aggregate. This fact suggests then that other cues are responsible for the induction of this behavior. In this work, we have tested the hypothesis that direct contact between non-overwintering individuals stimulates the establishment of clusters. Binary choice experiments highlighted the involvement of elytral cuticular compounds in this phenomenon. Chromatographic analyses showed that the active extracts contained mainly hydrocarbons, including saturated, mono-unsaturated, and di-unsaturated homologues. Physical contact also seems to be involved in the non-overwintering aggregative behavior of *H. axyridis*, but to a lesser extent than these natural compounds. These findings could eventually be used to develop new control methods of these pest populations and so, reduce the adverse impacts it causes on biodiversity.

Keywords Multicolored Asian ladybeetle · invasive species · elytral compounds · hydrocarbons · intraspecific communication · non-overwintering aggregation

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Introduction

Aggregation is a common phenomenon observed in Coccinellidae. Indeed, in early autumn, they look for a shelter to overwinter where they congregate to survive unfavorable conditions (low temperatures and scarcity of food) (Hodek 1996). Many species present this behavior, including *Adalia bipunctata* (L.), *Coccinella septempunctata* (L.), *Ceratomegilla undecimnotata* (Schneider), *Harmonia axyridis* (Pallas), *Hippodamia variegata* (Goeze), and *H. convergens* (Guérin-Méneville) (Copp 1983; Hemptinne 1985; Honěk et al. 2007; Obata 1986). The multicolored Asian ladybeetle, *H. axyridis,* is a particularly interesting model species because its aggregations significantly disrupt human habitations (Sloggett et al. 2011). Because these exotic insects often aggregate inside dwellings during winter, in addition to inconveniences caused by their large numbers, this adaptive behavior can induce allergic reactions in the occupants (Goetz 2006; Nakazawa et al. 2007).

In most cases, aggregation is an active process resulting from two types of stimuli. The first type consists of responses of individuals toward heterogeneities in physical factors, such as light or temperature gradients and environmental irregularities (Fraenkel and Gunn 1961). The second comes from interactions between conspecifics, where each individual can attract others (Sempo et al. 2009). This attraction between congeners implies an intraspecific communication through chemical cues. Recently, it has been highlighted that overwintering *H. axyridis* uses substrate markings composed of long-chain hydrocarbons deposited by conspecifics to orient, at short distances, toward aggregation sites and ensure the cohesion of the cluster (Durieux et al. 2012).
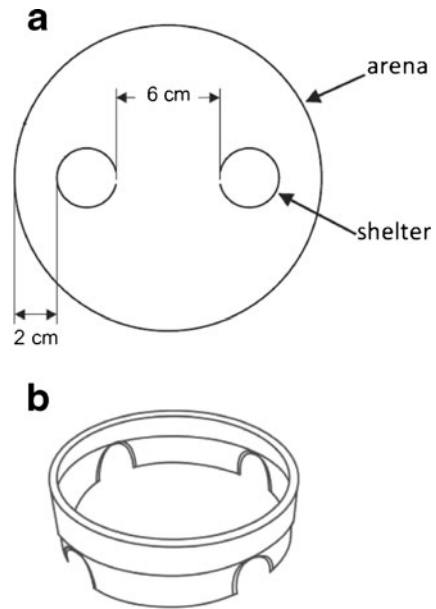
Although *H. axyridis* aggregation is described as a phenomenon typically related to the winter, this behavior can also be observed in non-overwintering conditions (personal observations). However, the substrate marking deposited by non-overwintering individuals does not seem to be used by *H. axyridis* in the same physiological state (Durieux et al. 2013). This suggests then the involvement of other cues in the formation of aggregations occurring during the non-winter period. In this work, the hypothesis according to which *H. axyridis* individuals are looking for contact with conspecifics by aggregating in non-overwintering conditions was investigated. Binary choice experiments were performed to test (1) the effect of physical contact and (2) the impact of cuticular elytral compounds on this exotic species. Moreover, chromatographic analyses were carried out to identify and quantify the chemicals that could be responsible for the observed behavior.

Materials and Methods

Biological Material

Multicolored Asian ladybeetles were collected from infested dwellings in Gembloux (Belgium) during Winter 2010. As cuticular compounds profiles may be age-specific (Everaerts et al. 2010), we tested ladybeetles of the same age. Ovipositing by the collected ladybeetles was then induced by providing them with pea aphids, *Acyrthosiphon pisum* Harris. Hatched larvae were also supplied with *A. pisum,* and the newly emerged adults were placed in 36×15×8 cm aerated plastic boxes (± 20 individuals per container). Each box was provided with sugar lumps, a water-

impregnated sponge, and multiflower pollen. The non-overwintering ladybeetles obtained by this way were kept in a controlled environment maintaining a 16-h-light photoperiod, 24±1 °C temperature and 45±15 % RH.

Bioassays

The roles of both physical contact and cuticular compounds on the cohesion of an aggregation were tested in a binary choice experiment. The experimental set-up consisted of a circular glass arena (diameter: 18 cm, height: 4 cm) containing two shelters placed symmetrically around its center (Fig. 1a). The shelters consisted of transparent plastic Petri dishes, 4 cm in diameter and 1 cm high, turned upside down (Fig. 1b). Four holes, each 7 mm in diameter, were pierced in an equidistant manner around each dish to allow ladybeetles to enter the shelters.

Two densities of 5 weeks ladybeetles were studied: 1 and 20 individuals. When only one ladybeetle was tested, each individual was used only once. For the groups of 20 individuals, the batches were modified from one experiment to another.

Ladybeetles were released in the center of the glass arena previously cleaned with the liquid detergent RBS T 105 (Chemical Products R. Borghgraef, Brussels, Belgium), distilled water and norvanol (ether-denaturated ethanol: VWR International, Haasrode, Belgium); the number of individuals inside each shelter was then observed after 30 min, 1, 8 and 24 h. Thirty replicates were carried out for each density. To avoid any bias coming from the position of the shelters, their position inside the arena was inverted between each replicate. From one repetition to another, the shelters were also cleaned with RBS T 105, distilled water and norvanol. Control assays (n=10), conducted in such a glass arena device presenting a cleaned shelter and a never used one to one ladybeetle, were preliminary performed to verify the suitability of this cleaning system (Chi-square Goodness-of-Fit Test (α=5 %, 1 df); χ^2 varied from 3 to <0.001, P varied from 0.083

to >0.999). The experiments were performed under the following conditions: 24±1 °C temperature, 45±15 % RH and a constant luminosity of 2000±200 lx.

Physical Contact

To test the involvement of physical contact in *H. axyridis* aggregation, eight 7 mm diameter glass balls were placed inside one of the two shelters in order to increase its contact area. The balls were previously cleaned by immersion in a RBS T 105 bath for 24 h, then rinsed with distilled water and norvanol. After washing, they were only manipulated using tweezers, to avoid depositing any compounds from finger sebum (e.g. squalene). The second shelter was left empty. Preliminary tests had shown there was enough space under a shelter to contain eight glass balls and 20 beetles simultaneously.

Cuticular Compounds

To assess the possible role of chemical compounds covering *H. axyridis* elytra in the aggregation phenomenon, eight glass balls covered with elytral extracts were placed inside one of the two shelters. In order to take into account only the effect of the extracted compounds by eliminating any physical contact effect, eight clean glass balls were placed under the second shelter. The balls were cleaned with RBS T 105 and norvanol before being used in the test, and subsequently only manipulated with tweezers.

The chemical extracts were obtained by immersing eight elytra in 1 ml of n-pentane (analytical reagent grade, 99.7 % pure- Fischer Scientific, Loughborough, Leicestershire, UK) for 5 min under constant agitation. Elytra were pooled according to the sex and the morph of ladybeetles, in order to reduce inter-individual variations. The sex of the adults was determined according to the criteria described by McCornack et al. (2007). The extracted elytral compounds were then deposited on a ball by immersing the ball in the 1 ml-solution, and then evaporating the solvent under a fume hood. During the evaporation process, the vials containing both a ball and a chemical extract were placed on a wreathing platform beater (Polymax 1040, Bioblock Scientific Heidolph, Germany), so that each ball was uniformly coated. Large quantities of elytral compounds were lost by settling on the inner surfaces of the vial. However, the quantity deposited on each ball via this procedure was still equivalent to the amount naturally encountered on the elytral surfaces of two ladybeetles, the ball being approximately twice the surface of one individual. This fact was verified by extracting the compounds effectively deposited on balls through this method (n=5) and comparing the collected quantities with the ones estimated covering the elytra of two *H. axyridis* (14.68±1.08 µg C_{19} equivalent, mean ± SD). Each ball was used only once.

The eight control balls, placed in the second shelter, were prepared by immersion in 1 ml of straight n-pentane, and then left under a fume hood on the wreathing platform beater until the solvent had completely evaporated. This step was performed in order to avoid any bias coming from eventual residues left by the solvent on balls coated with the cuticular extract after evaporation.

Statistical Analyses on the Bioassay Results

A Chi-square Goodness-of-Fit Test (α=5 %, 1 df) was performed on data from the binary choice bioassays involving only one individual, to highlight any eventual

preference of ladybeetles for either shelter. The observed frequencies were compared to corresponding theoretical frequencies (equal proportions of ladybeetles choosing each shelter). At the 20 ladybeetle density, individual choices were influenced by the presence of congeners. So, a binary logistic regression ($n=30$, $\alpha=5$ %), with the function logit being used as link, was performed to compare the evolution of ladybeetle numbers present under each shelter. These two statistical analyses were carried out for both types of experiment: testing physical contact on the one hand, and elytral cuticular compounds on the other.

Thereafter, the influence of interactions between ladybeetles on their individual choice was assessed by comparing the proportions of sheltered *H. axyridis* through all replicates between the four behavioral experiments. Given that the observation "being under a shelter" produces binary data, a binary logistic regression ($n=4$, $\alpha=5$ %) was used to carry out this comparison, the function logit being also used as link.

On the other hand, the comparison between the compound quantity effectively deposited on a ball through our covering process and the amounts naturally encountered on the elytra of two *H. axyridis* was conducted by using two statistical analyses: (1) a one-way analysis of variance (ANOVA) ($\alpha=5$ %) on the total quantities of elytral compounds and (2) a one-way multivariate analysis of variance (MANOVA) ($\alpha=5$ %) to compare the chemical profiles.

All statistical tests were conducted using Minitab® 15.1.1.0. (State College, Pennsylvania USA).

Chemical Analyses

Quantification of Cuticular Compounds

Cuticular lipids were extracted by immersing one pair of elytra from a five week ladybeetle in 1 ml of n-pentane for 5 min under constant agitation. Before cutting off elytra, ladybeetles were killed by placing them at -80 °C for 24 h. N-pentane was chosen because it is one of the most efficient solvents for extracting hydrocarbons (Rivault et al. 1998), and it exhibits less penetrating power compared with chlorinated solvents, what enables to extract only cuticular compounds. It also has a very low boiling point ($BP_{760}=36.1$ °C), and evaporates easily at room temperature.

This extraction procedure was repeated ten times for each sex and morph (distinguishing melanic and non-melanic ones). The compounds were quantified using n-nonadecane as an internal standard (IS) (22 µg/ml). Preliminary analyses had confirmed the absence of this molecule in the cuticular extracts. Moreover, the standard selected has to be close chemically to the collected compounds, and the latter are likely hydrocarbons, given that such homologues were obtained by using this protocol on *A. bipunctata* (Hemptinne et al. 1998).

Each sample was analyzed by gas chromatography (GC) on a Thermo Trace Fast GC (Thermo Electron Corporation) 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 injection was performed at a split ratio of 20:1. The injector temperature was 310 °C; the carrier gas was helium (0.5 ml/min). The programmed temperature was 40 °C for 30 s, followed by a gradual increase of 60 °C/min to 310 °C, which was held for 1 min.

Identification

Given that the same peaks were obtained whatever the sex or morph of the individual *H. axyridis* (see Cuticular Compounds section), the remaining extracts from the quantification process and the verification of the coating procedure were pooled (72 ml) and concentrated to ±50 μg/ml under a gentle stream of nitrogen for identification purposes. The GC-MS (Gas Chromatography—Mass Spectrometry) investigations were performed on an Agilent Technologies 6890 N Network GC System equipped with an 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. The operating conditions were: split ratio of 20:1, injector at 300 °C; carrier gas: helium at 1.7 ml/min; temperature program: from 40 °C (held for 2 min) to 320 °C, increasing at 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 at 230 °C, transfer line at 320 °C, scanned mass range: 40 to 500 m/z).

   The detected peaks were identified by their characteristic fragmentation patterns. The identification of saturated compounds was then confirmed by injection of pure n-alkanes standard (from n-C_9 to n-C_{40}). To identify the positions of double bonds in monounsaturated compounds, an epoxidation using m-chloroperbenzoic acid was performed (Mallet et al. 1985). In this reaction, an epoxide is synthesized at the position of each double bond, causing the molecule break at this place in the mass spectrometer, thus yielding two fragments with characteristic masses. This reaction was performed on approximately 500 μg of hydrocarbons. First, n-pentane was evaporated at 50 °C in a Büchi Rotavapor R-114. Two hundred microliters of chloroform (Merck KGaA, Darmstadt, Germany) and 200 μl of a chloroformic solution of m-chloroperbenzoic acid (25 mg/ml) (Acrōs organics, New Jersey, USA) were then added to the residue. The resulting blend was continuously agitated for 2 h. After this, 200 μl of an aqueous solution of sodium bisulfite (50 mg/ml) (Acrōs organics, New Jersey, USA) and sodium bicarbonate (50 mg/ml) (Carlo Erba, Milano, Italy) were added, followed by 1 ml of chloroform. The resulting mixture was rinsed twice with 1 ml of distilled water and dried using anhydrous sodium sulfate (Merck KGaA, Darmstadt, Germany). Finally, the sample was kept at −18 °C until GC-MS analysis.

Comparison of Cuticular Chemical Profiles

A two-way MANOVA was performed on the evaluated quantities of each molecule, in order to eventually highlight any quantitative differences in cuticular composition between the two sexes or the two morphs (melanic and non-melanic). Given the small size of our samples, the Pillai trace was used in order to prevent false negatives (Morrison 2005). Thereafter, a principal component analysis (PCA) was performed to visualize the highlighted difference. Both statistical analyses were conducted using Minitab® 15.1.1.0.

Results

Physical Contact

A significant preference for the shelter containing balls was highlighted at 8 h, when a single beetle was placed in the bioassay (Table 1). When 20 ladybeetles were

Table 1 Data from the two binary choice experiments involving one ladybeetle. Number of individuals recorded in each shelter (i.e., the one testing either the physical contact or the elytral compounds, and the control) at each observation time and associated results of the Chi-square Goodness-of-Fit Tests, assessing the preference of *H. axyridis* for either shelter

Tested factor	Time (min)	Number of ladybeetles recorded inside the shelter[a]		χ^2 (1df)	*P* value
		Control shelter	Shelter testing the factor		
Physical contact	30	3	4	0.14	0.705
	60	5	7	0.33	0.564
	480	2	9	4.45	0.035*
	1440	3	10	3.77	0.052
Cuticular extracts	30	3	0	3	0.083
	60	6	1	3.57	0.059
	480	7	5	0.33	0.564
	1440	4	6	0.4	0.527

[a] Out of the 30 tested individuals

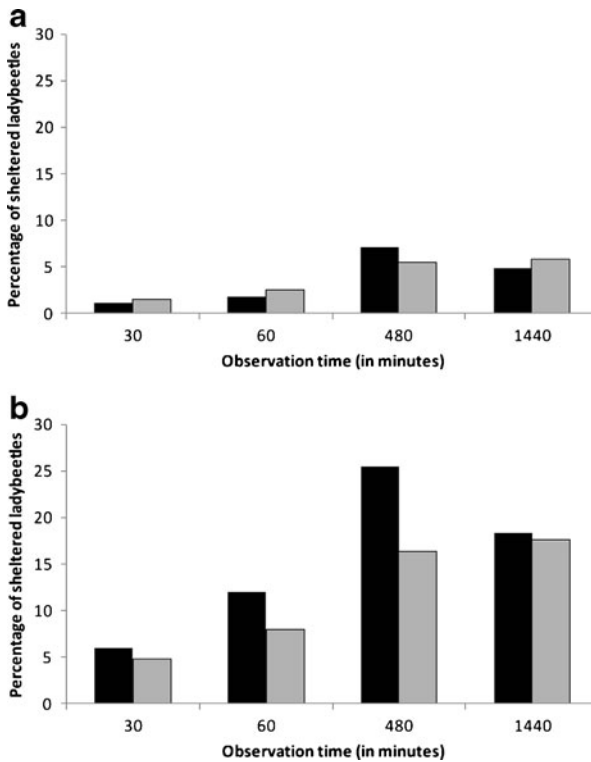*Statistical difference with $P<0.05$

Fig. 2 Results from the two binary choice experiments involving 20 ladybeetles. Comparison of the percentage of individuals recorded under the shelter testing the factor, i.e., (**a**) the physical contact and (**b**) the elytral compounds (*in black*) and under the control shelter (*in grey*) at the different observation times

introduced simultaneously, the binary logistic regression showed that the ladybeetles settled equally under both shelters (coef=−0.138, P=0.289) and the number of sheltered individuals increased as time passed (coef<0.001, P<0.001). A lower percentage of sheltered individuals is nevertheless recorded at 24 h experiment time in comparison to that obtained at 8 h (Fig. 2a). No interaction between the two factors (i.e., type of shelter and time) was observed (coef<0.001, P=0.808).

Cuticular Compounds

GC-MS analyses revealed that the cuticular extracts were made of the same 12 compounds whatever the sex or the morph of the *H. axyridis* individual, including five saturated hydrocarbons, six mono-unsaturated hydrocarbons, and one hydrocarbon with two double bonds (Fig. 3 and Table 2). The MANOVA showed that both males and females have a similar elytral chemical profile ($F_{8,29}$=1.10, P=0.391) but this profile was statistically different between the two morphs ($F_{8,29}$=2.35, P=0.044*). No interaction between the factors "sex" and "elytral color" was observed ($F_{8,29}$=0.82, P=0.595). The PCA resulted in two Eigen values>1 explaining 85.5 % of the total variance. Despite the difference highlighted between the two morphs by the MANOVA, the two dimensional plot of these first two components does not clearly show any difference between melanic individuals and non melanic ones (Fig. 4).

The statistical analyses conducted on the amounts extracted from coated balls confirmed that these quantities were equivalent to the ones naturally found on the elytra of two *H. axyridis* (ANOVA: $F_{1,43}$=2.82, P=0.100; MANOVA: $F_{7,37}$=1.79, P=0.119).

The bioassay, exposing a single individual to a choice between one shelter with clean glass balls and one containing glass balls coated with cuticular extracts, showed no preference for either shelter, for any given observation time (Table 1). On the other hand, when 20 ladybeetles were exposed simultaneously, they preferentially settled under the shelter containing balls coated with elytral compounds (coef=0.479, P<0.001) (Fig. 2b). An increase in the number of sheltered individuals was highlighted as time passed (coef<0.001, P<0.001) although a decrease in the percentage of sheltered ladybeetles was observed between the observation times of 480 and 1440 min (Fig. 2b). No interaction between the type of shelter and the time was observed (coef>−0.001, P=0.092).

Interactions Between Conspecifics

The total proportions of sheltered ladybeetles were similar in the two experiments involving one individual (coef=0.467, P=0.111) as well as in the bioassay observing
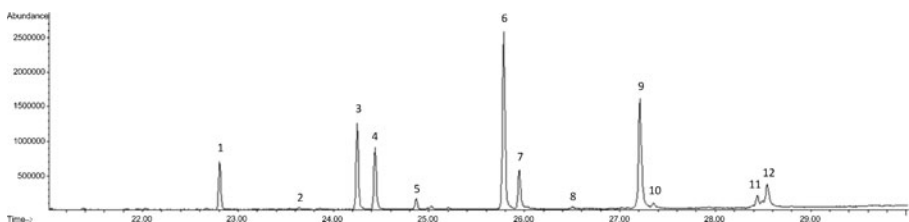

Fig. 3 Gas chromatogram of cuticular extract of *H. axyridis* elytra. Peaks are numbered according to the components identified by GC-MS in Table 2

Table 2 Identification and relative amounts of cuticular chemicals on a pair of *H. axyridis* elytra according to the sex and morph of the individual

Peak number[a]	Name	Abb[b]	RT[c]	M+[d]	Quantification (μg) (expressed in C_{19} equivalent) (mean ± SD)			
					Male		Female	
					Melanic	Non-melanic	Melanic	Non-melanic
1	n-Tricosane	nC_{23}	22.806	324	0.454±0.096	0.608±0.065	0.346±0.035	0.404±0.061
2	n-Tetracosane	nC_{24}	23.636	338	< LOD	< LOD	< LOD	< LOD
3	9-Pentacosene	$C_{25:1}$	24.250	350	0.924±0.237	1.270±0.223	0.634±0.126	0.875±0.163
4	n-Pentacosane	nC_{25}	24.435	352	0.678±0.153	0.773±0.076	0.535±0.046	0.514±0.082
5	Hexacosene[e]	$C_{26:1}$	25.032	364	< LOD	< LOD	< LOD	< LOD
6	9-Heptacosene	$C_{27:1}$	25.783	378	2.252±0.559	2.841±0.426	1.380±0.139	1.813±0.317
7	n-Heptacosane	nC_{27}	25.947	380	0.583±0.214	0.518±0.055	0.382±0.054	0.368±0.059
8	Octacosene[e]	$C_{28:1}$	26.503	392	< LOD	< LOD	< LOD	< LOD
9	9-Nonacosene	$C_{29:1}$	27.211	406	1.552±0.138	2.356±0.232	1.373±0.211	1.450±0.330
10	n-Nonacosane	nC_{29}	27.349	408	< LOD	< LOD	< LOD	< LOD
11	Hentriacontadiene[e]	$C_{31:2}$	28.438	432	0.395±0.049	0.675±0.111	0.510±0.099	0.785±0.075
12	9-Hentriacontene	$C_{31:1}$	28.544	434	0.584±0.081	0.906±0.101	0.472±0.034	0.550±0.099

LOD limit of detection

[a] The peak number corresponds to the peak identified on the gas chromatogram (Fig. 3)

[b] Abbreviation of hydrocarbon name

[c] Retention time

[d] Molecular ion

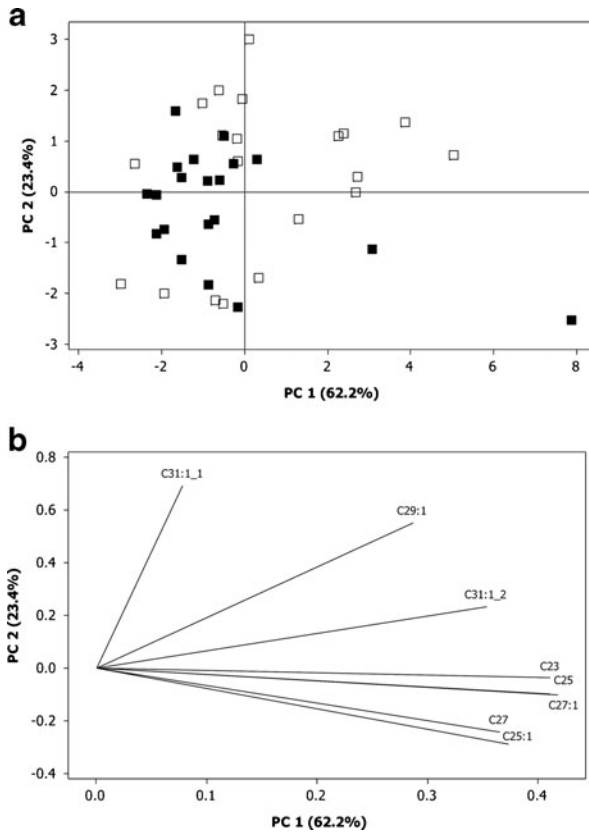[e] The positions of the double bonds were not established

Fig. 4 Principal component analysis of the cuticular chemical profiles of one pair of *H. axyridis* elytra according to the morph of the individual (non-melanic morphs (*white square*) and melanic ones (*black square*)). **a** Two-dimensional score plot of principal components one (PC1) and two (PC2) explaining 85.5 % of the variance in the data. **b** Load of each compounds in PC1 and PC2
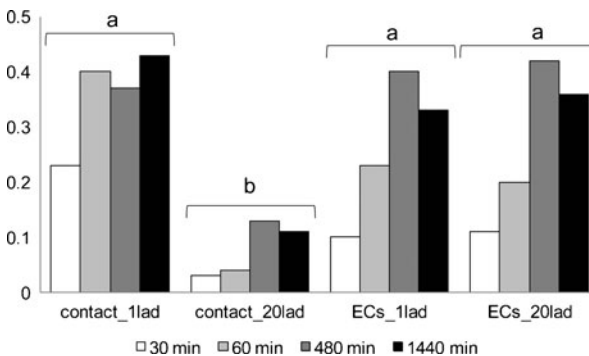

Fig. 5 Comparison of the total proportions of sheltered ladybeetles recorded at the different observation times between the four binary choice experiments: i.e., testing the impact of (1) physical contact on one ladybeetle (contact_1lad) and on 20 individuals (contact_20lad) and (2) elytral compounds on one ladybeetle (ECs_1lad) and on 20 individuals (ECs_20lad)

the behavior of 20 ladybeetles toward elytral compounds (coef=0.027, P=0.901). On the other hand, the total proportion of *H. axyridis* settling under one of two shelters was significantly lower when groups of 20 individuals had the choice between one empty shelter and one shelter containing balls without chemicals (coef=−1.559, $P<0.001$) (Fig. 5). These results were obtained by using Minitab, which selected the experiment testing the impact of physical contact on one ladybeetle as reference.

Discussion

Through this work, we highlighted the involvement of conspecific elytral cuticular compounds, made up of saturated and unsaturated hydrocarbons ranging from C_{23} to C_{31}, in the non-overwintering aggregation process of *H. axyridis*. Binary choice experiments showed a clear preference for shelters containing these chemicals, when groups of 20 individuals were tested. In contrast, no significant response was obtained for isolated ladybeetles. The mismatch between these results could be explained by a choice of the most suitable site occurring faster when a greater number of individuals are present. This hypothesis suggests the existence of a recruitment phenomenon set up by conspecifics that have found the artificial aggregation, consisting, in this case, of balls coated with cuticular compounds.

On the other hand, despite the fact that *H. axyridis* seems to present a positive physical contact response during its settlement inside overwintering sites by looking for crevices in which to aggregate (Hodek 1996), such a behavior was not clearly highlighted in the experiment performed. Indeed, a single positive response of a ladybeetle toward physical contact was observed at 8 h, when only one individual was tested. When a density of 20 individuals was used, no preference was underlined. This result could be due to a masking of the effect of physical contact by intraspecific interactions. In other words, thigmotaxis could intervene in this behavior but to a lesser extent than the cuticular chemicals. Interference by interattractivity between individuals is also supported by the difference observed in *H. axyridis* behavior when they are tested individually or in groups. Indeed, the total proportion of sheltered ladybeetles is lower when they were introduced by groups of 20 individuals in the set-up testing the involvement of physical contact. This observation can be explained by interactions existing between conspecifics which would slow down the exploration process of the device by ladybeetles. These intraspecific interactions could involve other chemical compounds than the elytral ones, such as the substrate markings highlighted in previous works (Durieux et al. 2012) or volatiles as suggested by Brown et al. (2006) and Verheggen et al. (2007).

Surprisingly, for assays involving elytral chemical compounds, no such interactions were observed on the ladybeetle choice. In fact, the total proportions of *H. axyridis* found under one of the two shelters was similar whatever the tested density. Moreover, in the majority of replicates, many individuals were found aggregated around or on the shelter containing the balls coated with elytral hydrocarbons. As the compounds coating the balls were non-volatile chemicals, these two facts strengthen the above assumption concerning the existence of a recruitment method set up by ladybeetles when they have found a suitable place to stay. The weak volatility of the tested extract also explains why no more than 43 % of ladybeetles were recorded under the shelters. Indeed, insects need to be in contact with these chemicals to detect them.

A recruitment process could be mediated by either non-volatile or volatile cues. The latter could be emitted by ladybeetles only when a suitable shelter is found. Such a phenomenon has already been observed in cockroaches. In this case, volatiles emitted by individuals that have found a profitable feeding resource are used by surrounding conspecifics to turn towards this site (Lihoreau and Rivault 2011). On the other hand, recruitment through the use of non-volatile chemicals could occur through the accumulation of substrate markings deposited by conspecifics around the appropriate site. The use of such cues in the localization of the nest entrance has been demonstrated in ants (Devigne and Detrain 2002) and wasps (Jandt et al. 2005).

The *H. axyridis* behaviors observed in our bioassays could nevertheless be somewhat influenced by thirst and/or hunger of individuals. Indeed, the results obtained from both behavioral experiments testing 20 individuals revealed a decrease in the percentage of sheltered ladybeetles between 8 and 24 h experiment time. This observation might be explained by the fact that the tested ladybeetles, being in an active stage, were not fed during the conducted assays. So after 8 h of bioassay, a number of individuals maybe leave the shelters looking for food and/or water.

This is the first report highlighting the involvement of elytral compounds in the aggregation behavior of ladybeetles. This discovery is in accordance with studies performed on other gregarious insects. In fact, several non-social insect species use information coded in cuticular hydrocarbons in their aggregation processes, including locusts (Heifetz et al. 1997) and cockroaches (Rivault et al. 1998; Said et al. 2005). It might also be interesting to study the behavior of *H. axyridis* towards extracts coming from other parts of the body (head, thorax, and abdomen), which would inform whether the aggregative behavior of *H. axyridis* is linked to hydrocarbons produced by all parts of the body, as is the case for the cockroach *Blattella germanica* (Rivault et al. 1998).

The chemical profile of *H. axyridis* elytra is the same whatever the sex of the individual. Moreover, no clear difference may be highlighted in the cuticular composition between morphs. This suggests that *H. axyridis* does not distinguish its conspecifics according to their sex or color feature during its aggregation behavior in non-overwintering conditions. This non-distinction is also observed in winter aggregations. Indeed, the *H. axyridis* clusters present in infested dwellings are made up of males and females belonging to several morphs (including melanic and non-melanic ones) (personal observations).

Introductions of *H. axyridis* in non-native areas, such as North America and Europe, have caused several environmental problems, including a decline of non-target species populations and injuries occasioned to fruits on which they feed before overwintering (Koch and Galvan 2008). The intraspecific interactions highlighted in this work as being involved in the formation of non-overwintering aggregations might then be used to develop new methods that would control *H. axyridis* movements in fields and therefore reduce the previously mentioned adverse impacts.

References

Brown AE, Riddick EW, Aldrich JR, Holmes WE (2006) Identification of (−)-beta-caryophyllene as a gender-specific terpene produced by the multicolored Asian lady beetle. J Chem Ecol 32:2489–2499

Copp NH (1983) Temperature-dependent behaviours and cluster formation by aggregating ladybird beetles. Anim Behav 31:424–430

Devigne C, Detrain C (2002) Collective exploration and area marking in the ant *Lasius niger*. Insect Soc 49:357–362

Durieux D, Fischer C, Brostaux Y, Sloggett JJ, Deneubourg JL, Vandereycken A, Joie E, Wathelet JP, Lognay G, Haubruge E, Verheggen FJ (2012) Role of long-chain hydrocarbons in the aggregation behaviour of *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae). J Insect Physiol 58:801–807

Durieux D, Fassotte B, Vanderplanck M, Deneubourg JL, Fischer C, Lognay G, Haubruge E, Verheggen FJ (2013) Substrate marking by an invasive ladybeetle: Seasonal changes in hydrocarbon composition and behavioral responses. PLoS One 8 art. no. e61124

Everaerts C, Farine JP, Cobb M, Ferveur JF (2010) Drosophila cuticular hydrocarbons revisited: mating status alters cuticular profiles. PLoS One 5 art. no. e9607

Fraenkel GS, Gunn DL (1961) The orientation of animals: Kineses, taxes and compass orientation. Dover Publications Inc., New York

Goetz DW (2006) *Harmonia axyridis* ladybug hypersensitivity in clinical allergy practice. J Allergy Clin Immunol 117:S29

Heifetz Y, Boekhoff I, Breer H, Applebaum SW (1997) Cuticular hydrocarbons control behavioural phase transition in *Schistocerca gregaria* nymphs and elicit biochemical responses in antennae. Insect Biochem Mol 27:563–568

Hemptinne JL (1985) Les sites d'hivernation de la coccinelle *Adalia bipunctata* (L.) (Col., Coccinellidae) en Belgique. Acta Oecol/Oec Appl 6:3–13

Hemptinne JL, Lognay G, Dixon AFG (1998) Mate recognition in the two-spot ladybird beetle. *Adalia bipunctata*: role of chemical and behavioural cues. J Insect Physiol 44:1163–1171

Hodek I (1996) Dormancy. In: Hodek I, Honěk A (eds) Ecology of Coccinellidae. Kluwer Academic Publishers, Dordrecht, pp 239–318

Honěk A, Martinkova Z, Pekar S (2007) Aggregation characteristics of three species of Coccinellidae (Coleoptera) at hibernation sites. Eur J Entomol 104:51–56

Jandt JM, Curry C, Hemauer S, Jeanne RJ (2005) The accumulation of a chemical cue: nets-entrance trail in the German yellowjacket, *Vespula germanica*. Naturwissenschaften 92:242–245

Koch RL, Galvan TL (2008) Bad side of a good beetle: the North American experience with *Harmonia axyridis*. BioControl 53:23–35

Lihoreau M, Rivault C (2011) Local enhancement promotes cockroach feeding aggregations. PLoS One 6 art. no. e22048

Mallet G, Dimitriades C, Ucciani E (1985) Apport des techniques de dérivatisation à la séparation des esters d'acides gras par chromatographie gazeuse capillaire. Rev Fr Corps Gras 11–12:439–446

McCornack BP, Koch RL, Ragsdale DW (2007) A simple method for in-field sex determination of the multicolored Asian lady beetle *Harmonia axyridis*. J Insect Sci 7:1–12

Morrison DF (2005) Multivariate Statistical Methods, 4th edn. Brooks/Cole/Thomson Learning, Pacific Grove

Nakazawa T, Satinover SM, Naccara L, Goddard L, Dragulev BP, Peters E, Platts-Mills TAE (2007) Asian ladybugs (*Harmonia axyridis*): a new seasonal indoor allergen. J Allergy Clin Immunol 119:421–427

Obata S (1986) Determination of hibernation site in the ladybird beetle, *Harmonia axyridis* Pallas (Coleoptera, Coccinellidae). Kontyu 54:218–223

Rivault C, Cloarec A, Sreng L (1998) Cuticular extracts inducing aggregation in the German cockroach, *Blattella germanica* (L.). J Insect Physiol 44:909–918

Said I, Costagliola G, Leoncini I, Rivault C (2005) Cuticular hydrocarbon profiles and aggregation in four Periplaneta species (Insecta: Dictyoptera). J Insect Physiol 51:995–1003

Sempo G, Canonge S, Detrain C, Deneubourg JL (2009) Complex dynamics based on a quorum: decision-making process by cockroaches in a patchy environment. Ethology 115:1150–1161

Sloggett JJ, Magro A, Verheggen FJ, Hemptinne JL, Hutchison WD, Riddick EW (2011) The chemical ecology of *Harmonia axyridis*. BioControl 56:643–661

Verheggen FJ, Fagel Q, Heuskin S, Lognay G, Francis F, Haubruge E (2007) Electrophysiological and behavioral responses of the multicolored Asian lady beetle, *Harmonia axyridis* Pallas, to sesquiterpene semiochemicals. J Chem Ecol 33:2148–2155