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Aggregation behavior of *Harmonia axyridis* under non-wintering conditions

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Abstract   The invasive multicolored Asian ladybeetle, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae), aggregates inside dwellings during winter to avoid cold weather. This adaptive behavior disturbs homeowners, because of the large numbers of individuals that aggregate, which induces allergic reactions. The migratory flight patterns of this species have been well documented, with individuals preferentially moving toward prominent and high color contrast elements. However, the factors involved in the selection of aggregation sites by this species have yet to be elucidated. Here, we evaluated the influence of (i) the density of individuals and (ii) the type of available shelters on decisions by *H. axyridis* to settle and aggregate under shelters. A dual choice bioassay conducted in the laboratory demonstrated the presence of mutual attraction to conspecifics. We also found that individuals preferentially settled under red covered shelters compared to transparent shelters, and that the type of shelter outweighed the effect of social interactions among conspecifics. Moreover, this experiment was performed under non-wintering conditions, providing the first evidence that aggregative behavior in this species can also occur under those specific conditions.

Key words   group effect; invasive species; luminosity; multicolored Asian ladybeetle; shelter selection; social behavior

Introduction

Several groups of insects form aggregations in response to certain cues, including locusts (Gillett, 1972), barber bugs (Lorenzo Figueiras *et al.*, 1994), and cockroaches (Jeanson *et al.*, 2005; Leoncini & Rivault, 2005), as well as aquatic insects, such as whirligig beetles (Wilkinson *et al.*, 1995). This phenomenon generally results from individual responses toward two types of cues. The first type of cue is based on environmental

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heterogeneities, including light or temperature gradients and soil irregularities (Fraenkel & Gunn, 1961). The second type of cue is associated with social interactions, through information transfer between conspecifics; whereby, each individual attracts other individuals (Camazine *et al.*, 2001). Several costs may be linked to this behavior, including an increase in intraspecific competition (for food, space, and reproduction) (Davies, 1962; Major, 1978; Moller & Birkhead, 1993), predation risk (Vet & Dicke, 1992), and vulnerability to parasitism (Brown & Brown, 1986). However, many advantages are associated with living in groups. Group living facilitates a decline in the heat and water loss of individuals (Heinrich, 1981; Heinen, 1993; Dambach & Goehlen, 1999), as well as communication between conspecifics (Dall *et al.*, 2005). There is also a reduction in predation

risk; for example, aggregated individuals exhibit increased vigilance (Treherne & Foster, 1980). However, aggregative behavior only arises when the benefits outweigh the detriments.

Several coccinellid species exhibit aggregative behavior, including *Coccinella septempunctata* (L.), *Ceratomegilla undecimnotata* (Schneider), *Hippodamia variegata* (Goeze), and *Hippodamia convergens* (Guérin-Méneville) (Copp, 1983; Hemptinne, 1985; Honek *et al.*, 2007). These species aggregate in areas covered with grass or stones, on trees or in trunk crevices, to survive unfavorable conditions (Hodek, 1973; Hemptinne, 1985; Honek *et al.*, 2007). Some ladybeetle species also aggregate inside houses, including *Adalia bipunctata* (L.) and *Aiolocaria mirabilis* (Motschulsky) (Hodek, 1973; Hodek, 1996); however, *Harmonia axyridis* (Pallas), the multicolored Asian ladybeetle, has been predominantly found forming aggregations in dwellings for several years, following its introduction to several countries (Brown *et al.*, 2008).

Harmonia axyridis is native to south-east Asia. In the 20th century, it was introduced to North America and Europe to control aphid and coccid populations (Brown *et al*., 2008). However, some negative impacts on nontarget species rapidly appeared after its introduction (Koch & Galvan, 2008). This species is now considered to be a threat to biodiversity, fruit production (because it aggregates on and consumes fruit to obtain carbohydrates before overwintering), and human health (Koch & Galvan, 2008). Indeed, beyond the annoyance caused by the number of individuals found inside homes during winter, the ladybeetles release hemolymph when disturbed, which contains alkaloids. These alkaloids cause allergic reactions, including rhinitis, conjunctivitis, chronic cough, asthma, and urticaria (Durieux *et al.*, 2010; Sloggett *et al.*, 2011).

Overwintering aggregation of *H. axyridis* has been widely examined in recent years. This typical behavior was precisely detailed by Nalepa *et al.* (2005) who suggested a five-step overwintering aggregation behavior: (i) exodus from feeding sites and orientation to the macrosite, defined as the visual feature on the horizon toward which ladybeetles fly to aggregate; (ii) landing on the surface of the macrosite; (iii) the decision to stay or leave, based on substrate features; (iv) the search for a microsite (i.e., a shelter in which the ladybeetles will take refuge, like window frame crevices); (v) settlement in the selected microsite. The first two steps have been well documented (Voronin, 1965; Nalepa *et al.*, 2000; Zenyoji, 2008). In addition, *H. axyridis* presents "hypsotactic" behavior, meaning that ladybeetles head for prominent isolated objects on the horizon (Obata, 1986). The species also shows a preference for sites with high color contrast (Nalepa *et al.*,

2005) but information about microsite selection remains limited.

In this work, we set out to record if the mechanisms involved in overwintering aggregation also appear under non-wintering conditions (Jeanson & Deneubourg, 2009). This issue was never investigated for the multicolored Asian ladybeetle, so the current study should highlight interesting and innovative results related to the chemical ecology of *H. axyridis*. Under those specific conditions, we studied the influence of (i) conspecific density and (ii) shelter luminosity on the aggregative behavior of *H. axyridis* in binary choice experiments conducted in the laboratory. To date, relatively few studies have examined the interplay between social interactions and individual responses to environmental heterogeneities. As *H. axyridis* is usually found to take refuge within frame window crevices, we designed an experimental set-up that enabled us to easily manipulate the characteristics of shelters. To our knowledge, binary choice experiments are the best option to evaluate the dynamics of aggregation and clearly establish a preferential choice. The findings of this study should provide important biological information related to the aggregative behavior of *H. axyridis*. Moreover, we expect that our results can contribute to elucidate the social interactions occurring between conspecifics. Through clarifying these factors, focused biocontrol methods may be developed to prevent this species from entering homes and forming aggregations.

Materials and methods

Biological material

The *H. axyridis* individuals were collected from natural habitats in Gembloux (Belgium) during the summers of 2009 and 2010. Adults were placed in $36 \times 15 \times 8$ cm aerated plastic boxes (± 20 individuals per container). Each box contained sugar, a water-impregnated sponge, and multiflower pollen. Boxes were placed in a controlled environmental chamber with a 16 h-light photoperiod, 24 ± 1 °C temperature, and $60\% \pm 5\%$ relative humidity (RH). These ladybeetles were then directly used in the experiments.

Binary choice experiment

The experimental set-up consisted of a circular glass arena (diameter: 18 cm; height: 4 cm) containing two identical shelters placed symmetrically on either side of the center (Fig. 1A). A luminosity of 1350 ± 150 lux was provided by two neon lights of 36 W, which were centered on the experimental arena. The shelters consisted
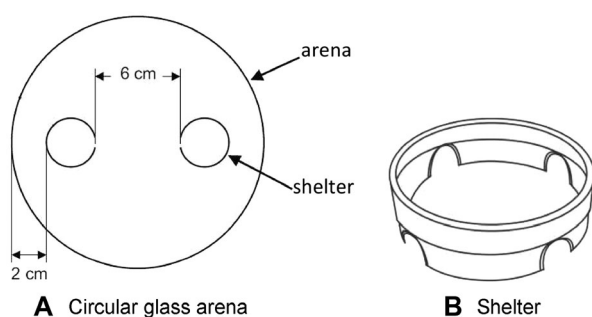
Fig. 1 Dual-choice bioassay. Experimental set-up used in the binary choice experiment. Diagram (A) represents the circular glass arena and (B) represents the shelter.

of plastic Petri dishes (diameter: 4 cm; height: 1 cm) that were turned upside down (Fig. 1B). Four holes of 7 mm diameter were pierced in an equidistant manner around each dish, to allow ladybeetles to enter the shelters. Ladybeetles were released centrally between the two shelters, and the number of individuals located inside each shelter was recorded several times after their release: every 10 min for the first hour, and then after 3, 5, and 8 hours. The experiments were performed under the following conditions: 8-h light photoperiod, $24 \pm 1\,°C$, and 60% $\pm$ 5% RH.

Three types of experiment were conducted using the previously described protocol. The first experiment involved two transparent shelters. The second experiment was carried out with two shelters covered with a red filter film (Amomask-4 transparent red), and the last one, combined a covered shelter and a transparent shelter. This last experiment is subsequently referred to as the "mixed assay." Both bioassays involving covered shelters were conducted to evaluate the effect of reduced brightness in shelters (125 ± 10 lux) on the behavior of *H. axyridis*. A red filter was chosen for two reasons: (i) *H. axyridis* appears to look for dark places to aggregate; and (ii) ladybeetles perceive red light as darkness, due to their lack of red light receptors (Lin, 1993).

In order to document any effect of the number of conspecifics on *H. axyridis* aggregative behavior, three densities of ladybeetles (1, 20, and 50 individuals) were tested for each type of experiment except for the mixed assay. For this assay, only two densities were considered (1 and 20 individuals), as the effect of an increase in number of conspecifics had already been studied in the two previous experiments. The trials involving only one individual were used as control. The shelters were designed to accommodate approximately 100 individuals, so that all the ladybeetles tested during one bioassay could be observed simultaneously under the same shelter. Thirty replicates

were performed per experiment and each individual was only used once.

Data analyses

The impact of the presence of conspecifics on the aggregative behavior of *H. axyridis* was estimated by using a generalized linear model (GLMs, binomial family and logit link) and its χ^2 lack of fit test on data from the experiment that tested two covered shelters. In this experiment, we offered a choice between two identical shelters, with it being assumed that the number of ladybeetles settled under one shelter should follow a binomial distribution if the choice of each ladybeetle is independent of other individuals. Conversely, aggregative behavior would result in greater dispersion than expected for binomial data, or overdispersion. However, direct adjustment test of the observations to a theoretical binomial distribution is not possible because the total number of individuals that actually choose a shelter differs between replicates and during the experiment, so that there is no unique distribution to match. But this overdispersion may easily be detected by the lack-of-fit test of our GLMs (Zirbes *et al.*, 2010). Since the experiment involved a choice between two identical shelters, the absence of any bias was tested using the same model, to ensure that the observed distribution was caused by aggregative behavior only.

To study the impact of shelter brightness, we compared the number of ladybeetles observed under the two transparent shelters versus the two covered shelters. Given that the observation of "being under the shelter" produces binary data, a binary logistic test (Minitab® 15.1.1.0, State College, Pennsylvania, USA; $n = 30$, $\alpha = 5\%$) was performed, with the function logit being used as link.

Results

Impact of conspecifics

The distribution of 20 and 50 individuals in the dual choice experiment was analyzed using GLMs, which revealed a stable significant difference between the observed distribution and a binomial distribution, from 3 h for 20 ladybeetles and from 20 min for 50 individuals. The results obtained for the observations of 20 and 50 individuals for each time-period are presented in Tables 1a and 1b, respectively. These results were not biased by the experimental set-up for any observation time (see *P* value (bias) in Table 1). The comparison between the two tables shows greater chi-squared values (χ^2) when density is superior, with the chi-squared parameter

Table 1 Statistical results of the bias and goodness of fit (GOF) tests of the generalized linear model comparing the distribution of ladybeetles that used covered shelters to a binomial distribution for each observation time and at two tested population densities: (a) 20 individuals, and (b) 50 individuals. Rejection of GOF test indicates a significant aggregative behavior in the corresponding assay.

(a) Observation time	Sheltered ladybeetles	*P* value (bias)	χ^2	*P* value
10 min	152	0.746	43.99	0.028[*]
20 min	243	0.481	38.70	0.107
30 min	315	0.612	50.88	0.007[**]
40 min	353	0.489	38.67	0.108
50 min	369	0.499	48.89	0.012[*]
1 h	382	0.413	40.31	0.080
3 h	489	0.190	68.63	<0.001[***]
5 h	502	0.284	59.61	<0.001[***]
8 h	515	0.355	66.65	<0.001[***]
(b) Observation time	Sheltered ladybeetles	*P* value (bias)	χ^2	*P* value
10 min	411	0.257	38.79	0.106
20 min	720	0.766	56.48	0.002[**]
30 min	912	0.354	93.93	<0.001[***]
40 min	1019	0.471	103.45	<0.001[***]
50 min	1068	0.088	97.15	<0.001[***]
1 h	1093	0.174	120.99	<0.001[***]
3 h	1199	0.260	165.49	<0.001[***]
5 h	1232	0.425	169.40	<0.001[***]
8 h	1243	0.755	215.89	<0.001[***]

*, **, and *** indicate statistical differences at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

quantifying the difference compared to a binomial distribution. Figures 2(A) and (B) present the comparison between the theoretical and observed distributions of the number of ladybeetles settled under the left dark shelter of the binary choice experiments using the observation time of 3 h as an example. As the total number of sheltered individuals varies between replicates, we used reduced proportions (i.e., centered by their theoretical mean and scaled by their standard deviation) to represent them on a common scale, for illustrative purpose only. This figure shows that greater dispersion was obtained at the density of 50 ladybeetles. Moreover, a constant significant difference appeared earlier for the largest density (Table 1).

Impact of shelter luminosity

Figure 3 shows the mean proportion of ladybeetles found under the two transparent shelters compared to those found inside the two red-covered shelters. A greater proportion of individuals were observed under the dark shelters. Moreover, the binary logistic regression shows a significant difference for the two tested factors, luminosity (coef = 3.300; $P < 0.001$) and time (coef = 0.004; $P < 0.001$) (Fig. 3), as well

as for the interaction between both (coef = 0.0013; $P < 0.001$). Therefore, ladybeetles were more prone to take refuge inside dark shelters, with the proportion of sheltering individuals increasing with time. Furthermore, this increase was greater when the shelter was covered with red film. Figure 4 presents this final observation, showing the evolution of the percentage of sheltering ladybeetles with time in the experiments using identical shelters. The slope of the curves was shallower for transparent shelters compared to the dark shelters. Figure 4 also shows that the temporal evolution of the percentage of individuals settled under covered shelters was the same for all ladybeetle densities. For the transparent shelters, this evolution was similar for groups containing one and 20 individuals, but not for groups containing 50 individuals. When testing 50 individuals, the percentage of sheltering beetles was more than twice as large compared to those obtained at the two lower densities (i.e., approximately 10% after 120 min for groups containing one and 20 individuals). This proportion corresponds to the result that would be expected by chance, given that the surface area inside the two shelters [$2 \times (2 \times \pi \times 2^2 + 1 \times 2\pi \times 2) = 75.41$ cm^2] represents about 10% of the surface area of the whole set-up ($2 \times \pi \times 9^2 + 4 \times 2\pi \times 9 = 735.13$ cm^2). The behavior of *H. axyridis* was also
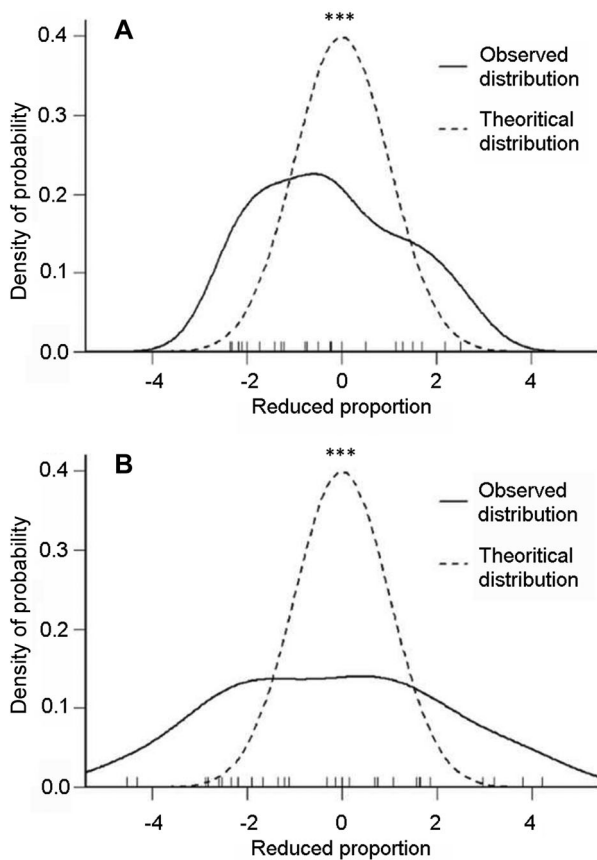
Fig. 2 Results of the generalized linear model after a 3 h observation time. Comparison of approximated theoretical (discontinuous stroke) and observed (continuous stroke) distributions of the number of ladybeetles inside the left dark shelter after 3 hours at two tested population densities: (A) 20 individuals and (B) 50 individuals (*** indicates statistical difference at $P < 0.001$). Bars at the bottom of the chart represent reduced proportions that were observed and used to calculate the density of probability.

compared between the "mixed assay" and the experiments using identical shelters. Figure 5 shows that the percentage of ladybeetles under a transparent shelter decreases if a covered shelter is also available. In contrast, the percentage increases if the ladybeetles only have access to two transparent shelters. Moreover, these two dynamics were observed when either one or 20 individuals were tested (Fig. 5).

Discussion

By comparing the actual distribution of groups of ladybeetles under two identical shelters with binomial distributions, we clearly demonstrated that social interac-
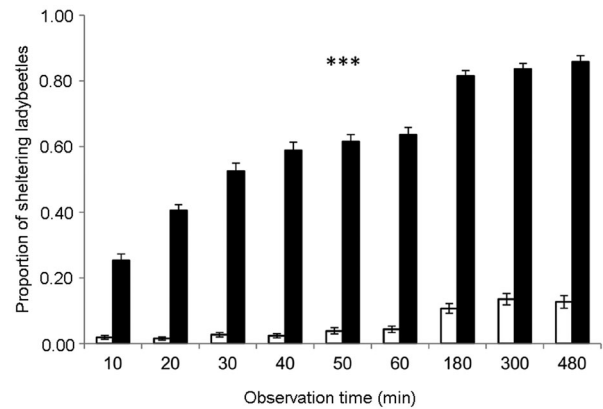
Fig. 3 Impact of shelter luminosity on the proportion of sheltering ladybeetles. Mean proportions of ladybeetles found under two transparent (white bars) and two dark (black bars) shelters for observation times ranging from 10 to 480 min (*** indicates statistical difference at $P < 0.001$). The results presented here were obtained using a group size of 20 ladybeetles, and the variation is expressed by the standard error ($n = 30$ replicates).
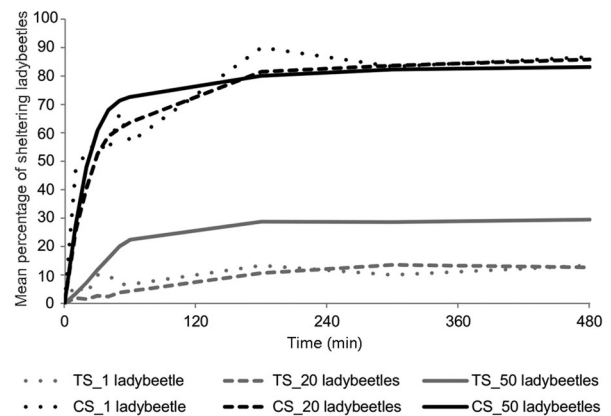

Fig. 4 Temporal evolution in the percentage of sheltering beetles. Evolution of the mean percentage of ladybeetles that sheltered under two transparent (TS) and two covered (CS) shelters over a 480 min observation period using three population densities (1, 20, and 50 individuals).

tions occur between *H. axyridis* individuals, and therefore the existence of an aggregative behavior in this ladybeetle species, even under non-wintering conditions (Jeanson & Deneubourg, 2009). This observation was further supported by the fact that the differences between the observed and theoretical distributions appeared more quickly, and were more pronounced, when the density of individuals was higher. This aggregative behavior does not solely arise because of environmental heterogeneities, as is evidenced by the unequal distribution of ladybeetles between the two identical shelters. This
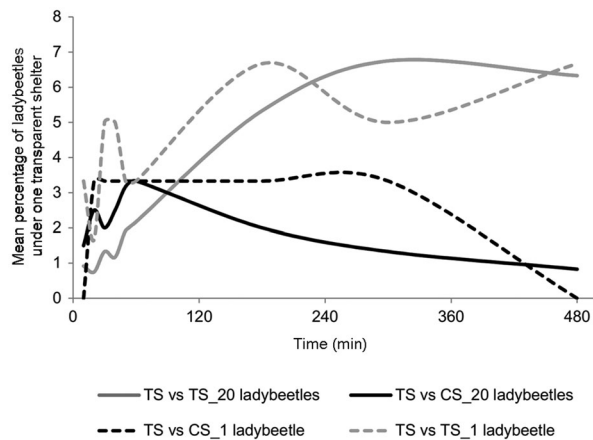
Fig. 5 Temporal evolution in the mean percentage of ladybeetles settled under one transparent shelter. Comparison of the data obtained from the "mixed assay" (black lines) with that obtained from the experiment using two identical transparent shelters (grey lines) ($n = 30$ and 60 replicates, respectively).

finding suggests the presence of interattractivity between individuals. Three hypotheses might explain this behavior: remote attraction, the retention phenomenon, or a combination of the two. Given that *H. axyridis* does not seem to be attracted by visual cues from conspecifics (Nalepa, 2007), volatile pheromones are probably involved. The existence of a molecule playing this role has not yet been identified with certainty in *H. axyridis*, although some authors have put forward the hypothesis of such a compound. For instance, Al Abassi *et al.* (1998) suggested that alkylmethoxypyrazines, might act as an aggregation pheromone in ladybeetles, in addition to their known olfactory alerting role. In fact, the attractant-arrestant capacity of 2-isopropyl-3-methoxypyrazine has been demonstrated in *C. septempunctata*, the 7-spotted ladybeetle. In addition, Brown *et al.* (2006) identified $(-)$-β-caryophyllene as a sesquiterpene emitted by the multicolored Asian ladybeetle under winter conditions which were recreated in the laboratory. Later, Verheggen *et al.* (2007) confirmed the attractant ability of this compound on *H. axyridis*, and suggested that this volatile intervenes in the aggregation process of this exotic species. Moreover, the retention phenomenon might involve physical contacts (thigmotactic behavior) or chemical compounds detected at short distances or by contact, which reinforce group cohesion. Recently, bioassays performed on the two-spotted ladybeetle, *A. bipunctata*, revealed that ladybeetles spent more time in the vicinity of an alkylmethoxypyrazine source when an extract containing cuticular hydrocarbons from diapausing individuals was present (Susset *et al.*, 2013). This finding indicates that

these molecules deliver information to ladybeetles searching for an aggregation site. Furthermore, studies on *H. axyridis* have demonstrated the use of substrate marking by this species during the aggregation process, in the localization and selection of overwintering sites (Durieux *et al.*, 2012). These markings, which are composed of saturated and unsaturated hydrocarbons, might also be involved in the behavior highlighted in this work. The effect of conspecific presence on the choice made by ladybeetles has also been observed for other coccinellid species. For instance, Honek *et al.* (2007) recorded a preference for certain overwintering sites in *C. septempunctata*, *C. undecimnotata*, and *H. variegata*, even though the sites seem identical.

When multicolored Asian ladybeetles reach a macrosite, they look for dark places, such as fissures or crevices, to overwinter in (Wang *et al.*, 2010). Thus, ladybeetles seem to be guided by negative phototaxis (Hodek, 1973). These observations were confirmed in the present work, as *H. axyridis* individuals preferentially settled under dark shelters. Nalepa *et al.* (2005) demonstrated that *H. axyridis* preferentially moves toward elements of high contrast during migratory flight. This phenomenon may explain why the number of *H. axyridis* under shelters increased faster when the shelters had a red covering. This faster increase could also rely on the modulation of individual behavior toward the quality of available shelters. For instance, if a ladybeetle spends more time under a dark shelter than under a transparent shelter, this longer resting time could facilitate the onset of aggregation under a dark shelter. Indeed, Jeanson and Deneubourg (2007) have reported that clustering depends on positive feedback associated with the presence of conspecifics; the probability of stopping and the duration of resting time both increasing with group size. In comparison, the transparent shelters did not attract *H. axyridis*, with the proportion of individuals found under these shelters corresponding to that expected if ladybeetles were uniformly distributed inside the arena (at densities of both one and 20 individuals).

Although social interactions were reported in the present work, it appears that visual features are more important to *H. axyridis* when selecting sites. When favorable (i.e., dark) shelters are available, the social effect is masked, whereas it was clear when transparent shelters were provided (Fig. 4). Moreover, the interaction between individuals was less significant compared to other gregarious insects. In similar binary choice experiments, the majority of cockroaches only selected one of two available shelters (Ame *et al.*, 2004; Sempo *et al.*, 2009). The same behavior was also documented for various densities of woodlice (Devigne *et al.*, 2011; Broly *et al.*, 2012). This behavior was not recorded in the current study, given

that the distributions were overdispersed in comparison to a binomial distribution, with absolutely no bimodal pattern (Fig. 2). This fact indicates that ladybeetles are not dependent on the choice of previous conspecifics, if this choice is not the most advantageous one. This hypothesis is strengthened by the observation that the proportion of ladybeetles under a transparent shelter only decreased when a more suitable resting site was available.

H. axyridis individuals are known to aggregate during winter to survive cold temperatures (Obata, 1986; Schaefer, 2003). In this work, we studied this behavior under non-wintering conditions. Although the non-uniform distribution of this exotic species in the field has already been highlighted (Koch, 2003), this study is the first to document the aggregative behavior of non-overwintering *H. axyridis* in the laboratory. According to the published literature, these aggregations seem to be related to the heterogeneous spatial distribution of prey; through (i) the attraction of these aphidophagous predators toward aphid honeydew or aphid pheromones (Sloggett *et al.*, 2011); and (ii) the trapping effect for predators, manifested by the time expense of eating prey and switching from extensive to intensive search effort after having consumed prey (Kawai, 1976). However, these facts do not preclude the existence of social interactions between individuals, even if these interactions are probably masked. Other coccinellid species also exhibit aggregated distributions in the field, including *Menochilus sexmaculatus* (Fabricius), *Coelophora inaequalis* (Fabricius), *Coccinella transversalis* (Fabricius), *H. convergens*, *Hippodamia quinquesignata* (Kirby), and *C. septempunctata* (Elliott & Kieckhefer, 2000; Evans & Toler, 2007; Rahman *et al.*, 2010). Hence, it might be assumed that social interactions between ladybeetle individuals, as highlighted in this study, also occur in these species.

Acknowledgments

Disclosure

The authors declare that there are no conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject of this manuscript.

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