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Synomones in necrophagous larvae of the blow flies *Lucilia sericata* and *Calliphora vomitoria*

Quentin Fouche¹  | Damien Charabidze^{2,3} | Christophe Lucas⁴

¹CHU Lille, EA 7367 - UTML - Unité de Taphonomie Médico-Légale, University of Lille, Lille, France

²UMR 8025, Centre d'Histoire Judiciaire, University of Lille, Lille, France

³Unit of Social Ecology (USE), Université Libre de Bruxelles (ULB), Bruxelles, Belgium

⁴Institut de Recherche sur la Biologie de l'Insecte (UMR 7261), CNRS - University of Tours, Tours, France

Correspondence

Christophe Lucas, Institut de Recherche sur la Biologie de l'Insecte (UMR 7261), CNRS - University of Tours, Tours, France.
Email: christophe.lucas@univ-tours.fr

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Abstract

Chemical signals are widespread in insects, but those resulting in interspecific communication (*i.e.*, synomones) remain understudied. Here, we analysed chemicals left on substrates by two species of blow fly larvae, *Lucilia sericata* (Meigen) and *Calliphora vomitoria* (Linnaeus) (Diptera: Calliphoridae), which can aggregate together on carrion. Using solid-phase microextraction and dynamic headspace analysis, we identified six compounds common to both species: the decanoic, tetradecanoic, pentadecanoic, hexadecanoic and octadecanoic acids, and the 2-ethylhexyl salicylate. We then tested the behavioural effects of the decanoic and pentadecanoic acids using binary-choice experiments, along with the (Z)-9-tricosene, a pheromone found in many arthropods. The time spent by a larva and its average crawling speed were measured in two sides of an arena, where only one contained a compound at 0.25 or 25 µg/µl. No effect was observed when testing the decanoic acid. The pentadecanoic acid only reduced the speed of *C. vomitoria* larvae at 25 µg/µl. Finally, *L. sericata* larvae spent less time in the side containing the (Z)-9-tricosene at 0.25 µg/µl, whereas *C. vomitoria* spent more time and crawled faster in this side at 25 µg/µl. Although these results did not directly evidence synomones, they suggest that the (Z)-9-tricosene could regulate larval aggregations on carrion.

KEYWORDS

(Z)-9-tricosene, aggregation, Calliphoridae, chemical communication, decanoic acid, dynamic headspace analysis, mixed-species groups, pentadecanoic acid, solid-phase microextraction, synomone

INTRODUCTION

Semiochemicals are the most widespread form of communication in insects. This chemical communication can occur between individuals of the same species (pheromones) or between different species (allelochemicals) (Brossut, 1997; Chapman, 2013). Allelochemicals are usually classified according to the beneficiary of the interaction: allomones benefit the emitter, kairomones benefit the receiver, while synomones benefit both (Chapman, 2013). In contrast to allomones and kairomones, studies examining synomones in insect-insect interactions are scarce. These compounds are mostly known in plant-insect interactions. For instance, they are released when some plants are attacked by phytophagous insects: by attracting predators, they

benefit both the plants and predators (Dicke & Baldwin, 2010). Yet, synomones may be involved in mixed-species groups, where each species can benefit from the presence of the others (Boulay et al., 2019; Komo et al., 2020). Such interspecific aggregations are common in necrophagous blow fly larvae, and maggots are therefore good candidates for identifying synomones.

Necrophagous blow flies breed on carrion: adults lay egg clutches on soft tissues, on which larvae feed and grow. While feeding, larvae gather in groups (maggot masses) that can include thousands of individuals from several species (Rivers et al., 2011). These aggregations facilitate larval feeding through heat generation (larval-mass effect) and exodigestion, increasing the development speed and the overall survival rate (Charabidze et al., 2011; Komo et al., 2020). Interspecific

larval aggregations result from eggs clustering, attraction towards food spots and high temperatures, but also from the chemicals produced by larvae on the substrate (Rivers et al., 2011). Under laboratory conditions, these compounds can attract and/or arrest either conspecific or heterospecific larvae (Fouche et al., 2018). Although the cuticular hydrocarbons and the organic compounds released by blow fly larvae have already been investigated (Frederickx et al., 2012; Gołębowski et al., 2012), the exact nature of chemicals that may act as cues for mixed-species groups formation remains to be determined.

The first aim of this study was to investigate the chemical composition of larval secretions left on substrate by two blow fly species, namely *Lucilia sericata* and *Calliphora vomitoria*, using two methods of Solid-Phase MicroExtraction (SPME) and a Dynamic Headspace Analysis (DHS). Second, the molecules identified in both species were used in binary-choice tests to assess their behavioural effects on larvae. To evidence a synomone, it was expected that at least one of these compounds induces a behavioural effect on either one or both species.

MATERIALS AND METHODS

Biological material

Wild *L. sericata* and *C. vomitoria* flies were collected in the surroundings of Lille (France). These species are widespread in Western Europe and can be observed simultaneously on the same carcass, forming interspecific maggot masses (Komo et al., 2019). Adult flies were reared separately at $25 \pm 2^\circ\text{C}$ under a natural daylight cycle with water and caster sugar *ad libitum*. To trigger egg laying, 50 g of fresh minced beef liver was given to adults for 2 h. After 5 days (*L. sericata*) or 7 days (*C. vomitoria*) of growth at $20 \pm 1^\circ\text{C}$ in the dark (Pol-Eko-Aparatura incubator model ST BASIC), newly moulted third instar larvae (8 ± 1 mm in length) were collected for experiments.

Extraction of larval secretions

The setup used for the extraction of the larval secretions was adapted from Fouche et al. (2018). Prior to any investigation, larvae were left at $25 \pm 1^\circ\text{C}$ in plastic boxes containing moistened pine sawdust for 4 h. This allowed any food remains from larvae to be avoided (see Fouche et al., 2018 for controls). Larvae were placed on a moistened filter paper (MP HYGIENE, France; 9 cm in diameter) at $25 \pm 1^\circ\text{C}$ for 10 min, so that the paper became impregnated by chemicals that larvae left while moving. After removing larvae, the paper was dry for 5 min under a fume hood and stored in a tied glass bottle at -20°C . Sixteen extractions (*i.e.*, 16 different papers impregnated of larval secretions) were performed in total: six with 40 *L. sericata* larvae, six with 40 *C. vomitoria* larvae, two with 80 *L. sericata* larvae and two with 80 *C. vomitoria* larvae. Additionally, several controls were performed: six control papers were treated with the same protocol but without larvae; six control glass

bottles without papers were let opened in the experimental room during the sample collection; and two control glass bottles without papers were left opened in the machine room just before the chemical analyses.

Chemical analyses

The samples were analysed using three different chemical processes to cover a wide range of candidate compounds: two Solid-Phase MicroExtraction (SPME) fibres coated with either (i) a polyacrylate phase (85 μm ; white; Supelco) or (ii) a Divinylbenzene/Carboxen/Polydimethylsiloxane phase (50/30 μm ; grey; Supelco), and (iii) a dynamic headspace analysis (DHS) using Tenax TA cartridges (Gerstel, 013741-005-00). Analyses were done using helium as carrier (1 ml/min) on an Agilent 7890B gas chromatograph coupled with an Agilent 7000C triple quadrupole mass spectrometer (GC-TQ) with a Gerstel MPS autosampler (Mülheim an der Ruhr, Germany), monitored by the software MassHunter (v.B07). The GC was equipped with a capillary column 30 m \times 320 μm \times 0.25 μm (Zebtron ZB-5HT Inferno). The oven temperature ramped from 30–320 $^\circ\text{C}$ at 10 $^\circ\text{C}/\text{min}$. The electronic impact was set at 70 eV in scan mode every 0.3 s with a 0.1 s scan lapse and scanned for 40–600 amu.

For SPME analyses, samples were incubated with fibres in tied glass vials heated at 30 $^\circ\text{C}$ during 0.5 min in a 500-rpm stirrer. The extraction lasted 180 min and was desorbed at 320 $^\circ\text{C}$ (white SPME) or 270 $^\circ\text{C}$ (grey SPME) during 300 s in the Thermal Desorption Unit (TDU-Gerstel) connected to a CIS4 injector. The initial temperature of the injector was -20°C (allowing cryo-focalization of the compounds) and increased to 330 $^\circ\text{C}$ (white SPME) or 280 $^\circ\text{C}$ (grey SPME) at 12 $^\circ\text{C}/\text{s}$, then hold for 1 min. The injector was set in split mode at a 15:1 ratio with an 18 ml/min split flow. The oven temperature started at 30 $^\circ\text{C}$ for 2 min, then was increased to 330 $^\circ\text{C}$ (white SPME) or 280 $^\circ\text{C}$ (grey SPME) at a rate of 5 $^\circ\text{C}/\text{min}$ and held for 1 min at these temperatures, respectively. After use, SPME fibre were conditioned following manufacture recommendations, *that is*, 2 h at 300 $^\circ\text{C}$ for white SPME or 1 h at 270 $^\circ\text{C}$ for grey SPME. For DHS analyses, samples were incubated in tied glass vials at 28 $^\circ\text{C}$ during 2 min in a 500-rpm stirrer. Headspace extractions were done under a 25 ml/min nitrogen flow during 30 min at 28 $^\circ\text{C}$ (transfer line at 150 $^\circ\text{C}$). The Tenax cartridges containing adsorbed compounds were then desorbed in the TDU with an initial temperature of 25 $^\circ\text{C}$ for 0.1 min, then temperature was increased to 300 $^\circ\text{C}$ at a rate of 100 $^\circ\text{C}/\text{min}$ and held for 2 min. The initial temperature of the connected injector was -20°C before to increase to 320 $^\circ\text{C}$ at a rate of 10 $^\circ\text{C}/\text{s}$ then held for 2 min. The injector was setup in solvent vent mode with a purge flow to split vent of 25 ml/min at 3.1 min and a vent flow of 50 ml/min. Samples were injected randomly within each extraction method.

By comparing chromatograms of the samples with the controls (*i.e.*, control papers and vials) we selected candidate compounds for behavioural experiments. Identifications of the candidates were done

using fragmentation patterns of their mass spectra compared to the NIST library 2011, current state-of-the-art and available chemical standards (McLafferty & Tureček, 1993; Nelson & Blomquist, 1995).

Behavioural experiments

Behavioural tests were designed to study the effect of the identified compounds on larval behaviour. The experimental setup was adapted from Fouche et al. (2018). As these tests opposed two different conditions, they are referred as “binary-choice test”; however, locomotory data were recorded rather than a simple choice. In brief, a glass Petri dish (2 cm in height, 9 cm in diameter) was divided into two equal semi-circles, filled with an agar solution (6%, 1 cm in height) that prevented drying and facilitated larval locomotion. Experiments were performed at 25°C (Pol-Eko-Aparatura incubator, model ST BASIC) under red light (630 nm). The absence of bias in the setup was checked by analysing larval behaviour in three control combinations (see Supporting Information for details, Table S1 and Figure S1).

Two hundred microlitres of solvent were spread on each semi-circle of the filter paper (hexane, >97%, Sigma-Aldrich, St Louis, USA). One semi-circle of the arena was treated with solvent only, while the other was treated with a solution comprising one of the three compounds of interest at either 0.25 or 25 µg/µl. The paper was then dried for 5 min under a fume hood and placed in the petri dish on the top of the agar solution. A naive larva was then placed on this paper at the centre, parallel to the medium line between both semi-circles, and tracked from above for 5 min (Canon EOS 750D camera). Between each test, devices were turned 180° so that each semi-circle was alternately left and right.

Three compounds were purchased and tested: (i) decanoic acid (>99%, Thermo Fisher, Kandel, Germany), (ii) pentadecanoic acid (>99%, Thermo Fisher, Kandel, Germany), (iii) and (Z)-9-tricosene (>98%, Tokyo Chemical Industry, Tokyo, Japan) (see the Discussion section for the reasons explaining the choice of these compounds). Each compound was dissolved into the solvent (hexane) at two concentrations: 0.25 µg/µl and 25 µg/µl. These concentrations were chosen based on previous studies testing those same compounds on other arthropod species (Xiao et al., 2010; Zhang et al., 2003). The solution was mixed into a vortex for 5 s before being deposited on the filter paper. In total, six binary-choice combinations were tested on both species using 25 replicates for each species in double blind conditions.

Data analysis

Data were recorded and analysed blindly regarding the treatments. For each binary-choice test, coordinates of the larva in the whole arena were measured every four seconds. The total time spent by the larva on each side of the device and its average crawling speed were calculated (the average crawling speed was calculated only

when larvae spent 15 s or more in the semi-circle; Fouche et al., 2018). Data were compared between both semi-circles using a Student's *t*-test (mentioned by “*t*” in the results) for paired data where normality and homoscedasticity permitted or using a Wilcoxon's test otherwise (mentioned by “*W*”). All analyses were performed with the R software (v3.6.2) with a significance level set at $\alpha = 0.05$.

RESULTS

Chemical analyses

DHS analyses revealed five acids common to both species (Figure 1): decanoic, tetradecanoic, pentadecanoic, hexadecanoic and octadecanoic acids, and another common compound, the 2-ethylhexyl salicylate. SPEM analyses detected in larval extracts of *L. sericata* the benzenethanamine, 3,4-dimethoxy- α -methyl- and the 3-buten-2-one, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl), and in *C. vomitoria* the 2,4-dimethyl-1-heptene.

Behavioural experiments

L. sericata larvae did not move faster nor spend more time in presence of the decanoic acid (0.25 µg/µl: time spent: $t = 0.43$, $p = 0.67$, average speed: $t = -0.83$, $p = 0.41$; 25 µg/µl: time spent: $t = -0.29$, $p = 0.77$, average speed: $W = 153$, $p = 0.95$; Figure 2). The same results were observed with *C. vomitoria* larvae at 0.25 µg/µl (time spent: $t = 1.67$, $p = 0.11$; average speed: $t = 0.55$, $p = 0.58$) as well as 25 µg/µl (time spent: $t = -0.63$, $p = 0.53$; average speed: $t = -1.39$, $p = 0.18$; Figure 2).

With the pentadecanoic acid at 0.25 µg/µl, nor the time spent neither the average speed of larvae were different between both sides of the arena in *L. sericata* (time spent: $t = 1.11$, $p = 0.28$; average speed: $t = -0.66$, $p = 0.51$) or *C. vomitoria* (time spent: $t = 0.22$, $p = 0.83$; average speed: $t = -0.73$, $p = 0.47$; Figure 2). At 25 µg/µl, the time spent did not differ between sides in both species (*L. sericata*: $t = -1.56$, $p = 0.13$; *C. vomitoria*: $t = -1.56$, $p = 0.13$). However, the average speed of larvae was significantly lower in the side containing the pentadecanoic acid than in the solvent-only treated side in *C. vomitoria* ($t = -2.75$, $p = 0.012$), but not in *L. sericata* ($W = 168$, $p = 0.61$; Figure 2b).

Finally, facing a choice between one side containing the (Z)-9-tricosene at 0.25 µg/µl and the other containing only solvent, *L. sericata* spent significantly less time in the (Z)-9-tricosene treated side ($t = -2.35$, $p = 0.027$), with no difference in average speed ($t = -1.20$, $p = 0.24$; Figure 2). In the same conditions, the time spent and average speed of *C. vomitoria* larvae did not differ between sides (time spent: $W = 210$, $p = 0.21$; average speed: $W = 173$, $p = 0.52$). The opposite result was observed at the highest concentration (25 µg/µl): while *L. sericata* larvae did not show any difference in time spent nor average speed (time spent: $W = 109$,

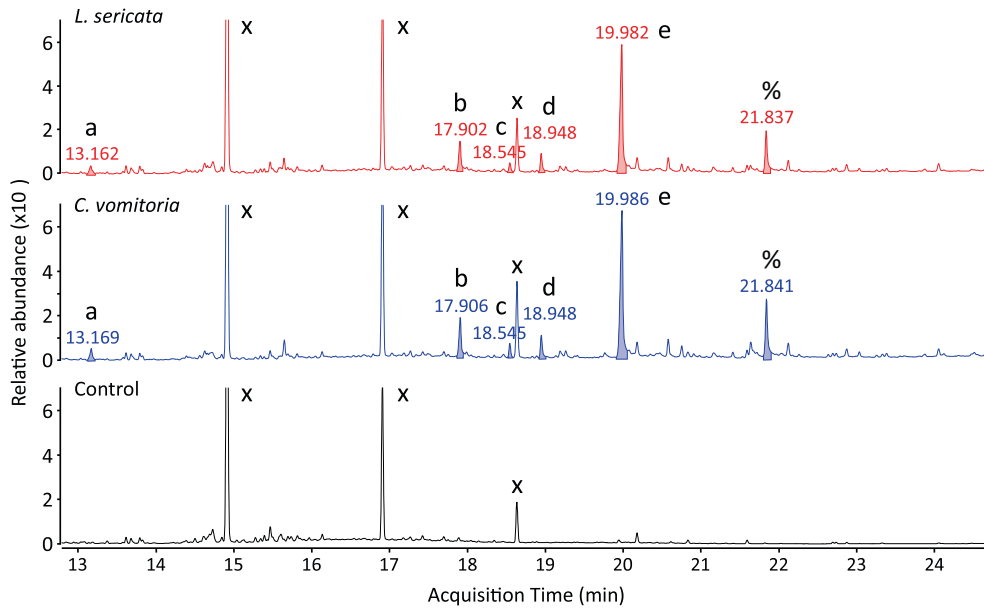


FIGURE 1 Chromatograms of larval extracts using the DHS method for *L. sericata*, *C. vomitoria* and control containing clean paper. The six compounds common to both species are highlighted with their retention times in the respective order: (a) decanoic acid, (b) tetradecanoic acid, (c) 2-ethylhexyl salicylate, (d) pentadecanoic acid, (e) hexadecanoic acid and (f) octadecanoic acid. The three peaks labelled “x” correspond to cyclosiloxane contaminants

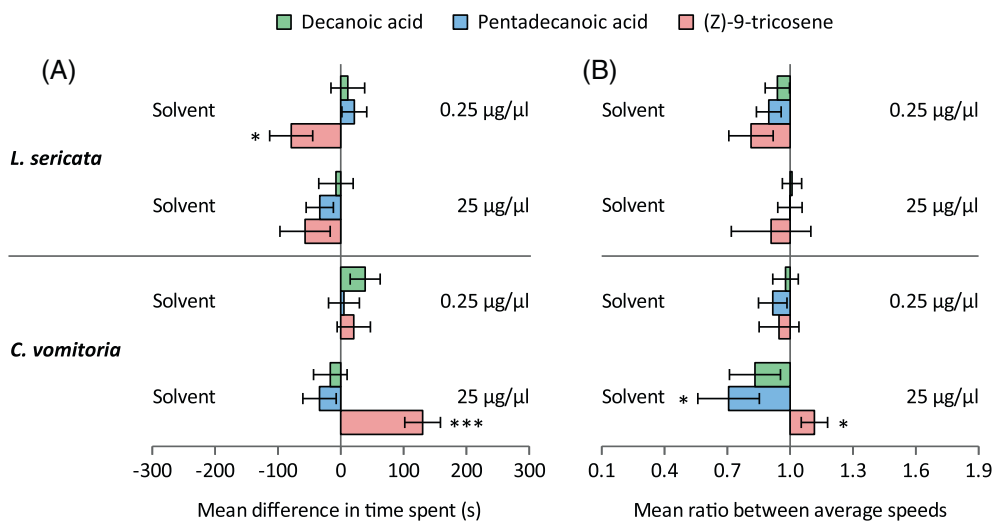


FIGURE 2 Time spent and average speed of larvae on each semi-circle with the tested chemical candidates. (a) Mean differences (\pm SE) in time spent in seconds and (b) mean ratio (\pm SE) between average speeds of *L. sericata* and *C. vomitoria* larvae on a solvent-only treated semi-circle and a semi-circle treated with one compound of interest (decanoic acid in green, pentadecanoic acid in blue, (Z)-9-tricosene in red). Concentration of the tested chemical candidates was either at 0.25 or 25 $\mu\text{g}/\mu\text{l}$ ($n = 25$ for each condition). To calculate the difference in time spent, the value on the compound-treated semi-circle was subtracted from the value on the solvent-only treated semi-circle. For the ratio between average speeds, this value was instead divided by the value on the solvent-only treated semi-circle. For example, a ratio of 0.5 means that larvae crawled two times faster on the solvent-only treated side than on the compound-treated side. Asterisks indicate a significant difference (Student's *t* test and Wilcoxon's test; * $p < 0.05$; *** $p < 0.001$)

$p = 0.15$; average speed: $t = 1.29$, $p = 0.21$), *C. vomitoria* larvae spent significantly more time and moved significantly faster in the (Z)-9-tricosene treated side (time spent: $W = 270$, $p < 0.001$; average speed: $t = 2.11$, $p = 0.048$; Figure 2).

DISCUSSION

We identified up to nine different compounds deposited on the substrate by the larvae of *L. sericata* and *C. vomitoria*. Among them,

six compounds were common to both species, with five acids and a salicylate. Interestingly, four out of the five acids (decanoic acid excepted) were also found among the compounds released by larvae of *C. vicina*, another closely related blow fly species, making them suitable synomone candidates (Frederickx et al., 2012). As it was not possible to test all the nine compounds we identified, we selected only two of them for behavioural assays. The pentadecanoic acid was tested as it elicits a strong aggregation effect (attractant and arrestant) in beetles (Cohen et al., 1974). We also tested the decanoic acid because of its attractive effect in beetles (Cohen et al., 1974) and because it presents an electrophysiologic activity in locusts (Torto et al., 1996). Finally, the (Z)-9-tricosene was also tested thanks to its well-known pheromonal action in arthropod species (Xiao et al., 2010; Zhang et al., 2003) and its presence in larvae of the blow fly *C. vicina* (Frederickx et al., 2012).

Neither the decanoic nor pentadecanoic acids affected the time spent by larvae on each side of the device. The lowest crawling speed observed in *C. vomitoria* on the semi-circle treated with pentadecanoic acid suggests a modification of larval exploration behaviour. However, this effect did not translate into an increase in retention time, which seems to be necessary to elicit an attractant effect, as previously observed in skin beetles (Cohen et al., 1974). The absence of effect on retention time could also indicate that the tested concentrations were not adapted to elicit a larval response in these species, which calls for more experiments with intermediate concentrations. Even though both acids do not seem to directly trigger or maintain aggregation, their presence in both *C. vomitoria* and *L. sericata*, as well as in *C. vicina* (Frederickx et al., 2012), suggests they could play a role in larval recognition and tolerance between species.

The most notable results of the behavioural experiments were observed when testing the (Z)-9-tricosene. This compound was not detected in our two tested species, but in both of them it induced a change in time spent by larvae in a side. At the highest concentration, an attractive/retentive effect (i.e., an increase in time spent in the (Z)-9-tricosene-treated side) was observed in *C. vomitoria*. If this compound is released by some species of blow flies (other than the two investigated in this study), then the attractive/retentive effect on *C. vomitoria* larvae could lead to the formation of interspecific aggregates on carrion (Boulay et al., 2019). When such aggregation provides mutual benefits, as already observed in some species (Komo et al., 2019, 2020), the (Z)-9-tricosene would play the role of a synomone. For now, more studies are needed on other blow fly species to confirm these assumptions. The (Z)-9-tricosene also induced an increase in *C. vomitoria* larval speed, which could reflect an increase in exploration activity. In *L. sericata*, the (Z)-9-tricosene was repulsive at the lowest concentration and neutral at the highest concentration. Such an effect could contribute to regulate aggregation by inhibiting it below a critical group size (i.e., a quorum; Sumpter & Stephen, 2009). Further studies need to be performed to confirm the presence of (Z)-9-tricosene in other blow flies, before drawing any conclusion on the role of this compound in larval aggregation.

Overall, this study has evidenced the ability of blow fly larvae to react to isolated chemicals, one they secreted (pentadecanoic acid)

and another one they did not release ((Z)-9-tricosene) but has a pheromonal effect in several other insect species. These findings do not support our initial hypothesis, that is, the existence of synomones that would trigger aggregation between different species of blow fly larvae. More studies are needed to either assess the presence of (Z)-9-tricosene in other blow fly species or test the other compounds that have been identified here. Further behavioural studies should also involve blends, as they are usually more likely to elicit a behavioural effect than isolated compounds (e.g., Torto et al., 1996; Zhang et al., 2003). Finally, the complexity of our results enforced the idea that the carrion is a complex structured environment, with multiple possible interactions and a hidden diversity of species and chemicals, yet to be fully discovered.

AUTHOR CONTRIBUTIONS

QF, DC and CL designed the experimental methodology. QF and CL collected and analysed the data. All authors wrote the manuscript.

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CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in figshare at <https://doi.org/10.6084/m9.figshare.14496207>.

ORCID

Quentin Fouche  <https://orcid.org/0000-0001-5507-7388>

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1. Time spent and average speed for *L. sericata* and *C. vomitoria* larvae in the control experiments. For each choice combination, values (mean \pm SE) are first given for the solvent-only treated semi-circle and then, in a second column, for the semi-circle treated either as blank (i.e., no solution added), with only solvent again, or with solvent plus 40 conspecific larvae ($n = 20$ for each condition). Test's values: *t* for Student's *t* test or *W* for Wilcoxon's test. Values in bold are statistically significant ($p < 0.01$ or 0.001).

Figure S1. Time spent and average speed of larvae on each semi-circle in the control experiments. A. Mean differences (\pm SE) in time spent in seconds and B. mean ratio (\pm SE) between average speeds of *L. sericata* and *C. vomitoria* larvae on a solvent-only treated semi-circle and a semi-circle either blank (i.e., no solution added), treated with solvent or treated with solvent plus conspecific larval cues ("40 Ls": cues left by 40 *L. sericata* larvae; "40 Cv": cues left by 40 *C. vomitoria* larvae). To calculate the difference in time spent, the value on the semi-circle treated as blank, with solvent or with solvent plus larval cues was subtracted from the value on the solvent-only treated semi-circle. For the ratio between average speeds, this value was instead divided by the value on the solvent-only treated semi-circle. Asterisks indicate a significant difference (Student's *t* test; ** $p < 0.01$; *** $p < 0.001$). Each condition was replicated 20 times.

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