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A first insight into the scanning behaviour of the presocial blow fly larvae

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Abstract. Aggregation of necrophagous larvae has several benefits: the sharing of salivary enzymes (exodigestion), temperature regulation, protection from predators and parasites, etc., and is well developed in blow flies (Diptera: Calliphoridae). The present study focuses on the aggregation mechanism used by the necrophagous larvae of *Lucilia sericata* Meigen, the common green bottle fly. The ability of single larva to detect and follow a signal (trail) created by conspecifics is investigated initially. A circular ring is drawn in a Petri dish where 20 starved larvae have crawled for a period of 30 min. A naïve (test) larva is then placed in the dish and video-tracked. Naïve larvae are able to detect the boundaries of the larvae-crawled area and stay preferentially within this conspecific-marked zone. In a second step, the orientation of larvae by scanning, a dedicated, ground-signal detection behaviour of dipteran larvae, is analyzed. Four experimental conditions are tested: control, presence of food, conspecifics, and food + conspecifics. When conspecifics have been previously present in a given area, the scanning behaviour by naïve larvae in this area decreases (both in number and frequency of scans). Accordingly, scanning by necrophagous larvae of *L. sericata* should be considered not only as locomotion behaviour, but also as a potential way to detect signals from conspecifics for the purpose of aggregation. The chemical composition of the attractant(s), the behavioural effects (attraction or retention) and the implication of larval signalling in the aggregation process are new fields to explore.

Key words. Aggregation, chemo-detection, self-organization, sensory organs, trail-following.

Introduction

Aggregation, or interattraction, is a common behaviour that occurs in many biological systems (Krause & Ruxton, 2002). With regard to necrophagous species, the costs and benefits of aggregation by blow fly larvae are identified and discussed by Ives (1991), as well as by Rivers *et al.* (2011). One main advantage is the capacity of large larval masses to locally modify some of the biotic and abiotic factors of their ecosystem, leading to faster larval development. The most well-known benefit of aggregation in necrophagous larvae is the local elevation of temperature inside aggregates, called the 'larval-mass effect' or, more familiarly, the 'maggot-mass effect' (Slone & Gruner, 2007; Hückesfeld *et al.*, 2011; Rivers *et al.*, 2011; Charabidze *et al.*, 2011). This heat generation accelerates the development of blow flies larvae and thus decreases the time spent by larvae on carrion (Heaton *et al.*, 2014; Johnson & Wallman, 2014). Gregarious behaviour also offers protection from predators and parasites, cooperation for digestion and an increase in food assimilation (Rivers *et al.*, 2011). Accordingly, gregarization appears to be a key behaviour in these species, which is especially remarkable because larval masses are self-organized structures: each larva has only a local perception of its close environment and (re)acts for itself (Camazine *et al.*, 2001).

Such social organization requires at least a basic communication system between individuals (Camazine *et al.*, 2001). In many social insect species, such as Lepidoptera or Hymenoptera, larval aggregations are mediated by tactile and/or chemical cues (Wertheim *et al.*, 2005; Costa, 2006). However, there are only a few studies investigating the

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mechanisms underlying the collective behaviour of blow fly larvae (Rivers *et al.*, 2011; Boulay *et al.*, 2013). In a recent study, the present research group (Boulay *et al.*, 2013) highlighted for the first time the existence of a signal passively deposited or left passively by *Lucilia sericata* larvae on the substrate over which they were crawling: these chemical cues were shown to have an attractive/retentive effect on conspecifics (Boulay *et al.*, 2013). These chemical cues are shown to have an attractive/retentive effect on conspecifics (Boulay *et al.*, 2013). The present study focuses on the behavioural response of *L. sericata* larvae to this signal, and particularly with regard to orientation mechanisms.

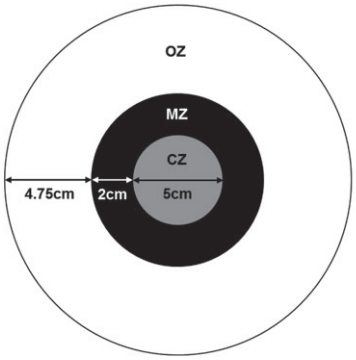
Green *et al.* (1983) describe five elementary behaviours considered to be shared by all cyclorrhaphous dipteran larvae (i.e. species where the imago emerges from the pupal case by pushing a lid or a circular seal). These are locomotion, turning, burrowing, rearing and bending. Rearing is defined as the raising and trembling of the head and first thoracic segments, whereas bending is described as the lateral flexing of the head and anterior segments (Green *et al.*, 1983). In other words, rearing occurs in a vertical axis and does not directly affect larval trajectory, whereas bending is laterally oriented. Accordingly, Green *et al.* (1983) assume that bending is mainly associated with changing direction (i.e. turning) during locomotion. However, observations made in our laboratory (J. Boulay & D. Charabidze, unpublished observations) strongly suggest that larvae may also use bending to detect the chemical signals around them and to orientate accordingly. Indeed, the anterior part of the larva's body is the location of most of the sensory organs: Calliphoridae larvae have 12 photoreceptors (Bolwig's organ) (Hinnemann *et al.*, 2010), as well as one olfactory and one gustatory organ (Chu & Axtell, 1971a,b; Cobb, 1999), all located in the anterior section. Bending could therefore be involved in local signal detection and should be regarded as a scanning (i.e. 'a mechanism by which animals move their receptors, and sometimes their bodies or appendages, so as to capture information from the environment efficiently') (Bell, 1990). Such a behaviour has been reported for many insect species during foraging, or when searching for conspecifics, refugees or mates (Bell, 1990).

To test this hypothesis, the present study first analyses the ability of *L. sericata* larvae to detect and follow a track previously crawled by conspecifics (Larval trail experiment). It then focuses on the scanning behaviour, which is hypothesized to be involved in the detection of the larval signal. To test the hypothesis, the scanning behaviour of *L. sericata* third-instar larvae is analyzed under four different experimental conditions: control, conspecific larval signal, food and larval signal + food together (Boulay *et al.*, 2013). According to this hypothesis, an increase of scanning behaviour should be observed in the absence of larval signal, demonstrating active searching for the conspecific's signal (i.e. chemotaxis) (Gomez-Marin *et al.*, 2011).

Materials and methods

Insect rearing

The experiments were performed on *L. sericata* larvae that were obtained from rearing colonies (Lille, France). Adults were reared at ambient temperature ($25 \pm 2\,^{\circ}C$) under natural light and fed *ad libitum* with caster sugar and water. Beginning with the adult fly emergence (day 0), fresh minced beef liver was added for 7 days (day 7) to provide the proteins necessary for vitellogenesis. After 5 days with no food, liver was provided again to trigger egg-laying (day 12). Eggs were placed on breeding substrate (100 g of fresh minced beef liver) at $17 \pm 0.5\,^{\circ}C$ (day 13). Larvae, 5–6 days old (days 18–19, corresponding to young third instars, 10 ± 2 mm long) were used for experiments (Grassberger & Reiter, 2001). During the experiments, the temperature of the set-up was maintained at $25 \pm 1\,^{\circ}C$ in a thermostatic chamber.



	Area (cm²)
Centre Zone (CZ)	19.63
Marked Zone (MZ)	37.11
Outer Zone (OZ)	212.06
Total	268.60

Fig. 1. Apparatus used for analyzing behavioural responses to the attractive signal from necrophagous larvae of the Green bottle fly *Lucilia sericata* (Control and Marked trials). Third-instar *L. sericata* larvae were placed in the Centre Zone (CZ). Trials started when individuals entered the Marked Zone (MZ) and stopped when larvae stayed for more than 1 min completely in the Outer Zone (OZ).

Larval trail experiment

This experiment was designed to test the ability of *L. sericata* larvae to follow a track created by conspecifics (i.e. a trail). The set-up consists of a Petri dish (diameter 18.5 cm; surface 268.60 cm^2) (Fig. 1) lined with a wet paper towel (humidified with distilled water) covering the arena surface. Two smaller Petri dishes were used to circumscribe a ring (internal diameter 5 cm, external diameter 4.75 cm; thickness 2 cm, surface 37.11 cm^2) (Fig. 1). Under the 'Control' condition, no deposit was made in this ring. The larval trail condition was obtained with 20 starved larvae (cleaning and starving took place for 4 h in wet pine-wood dust; a time sufficient to obtain larvae with empty crops; Charabidze *et al.*, 2013) crawling in the ring area for 30 min to create the Marked Zone (MZ) (Fig. 2). After 30 min, these 20 larvae and the two small Petri dishes were removed. A naïve (test) larva was placed in the centre of the arena [Centre Zone (CZ): 19.63 cm^2] (Fig. 1) in the dark and video recorded using an infrared camera (Kam-HWI-SH-7204; Kamatec, Germany). The results were analyzed with ETHOVISION, version 8.0 XT (Noldus, The Netherlands): trials started when the test larvae exited the CZ for the first time and were stopped when individuals were outside the MZ for at least 1 min or when they had reached the wall of the arena. Twenty replicates were performed for each condition.

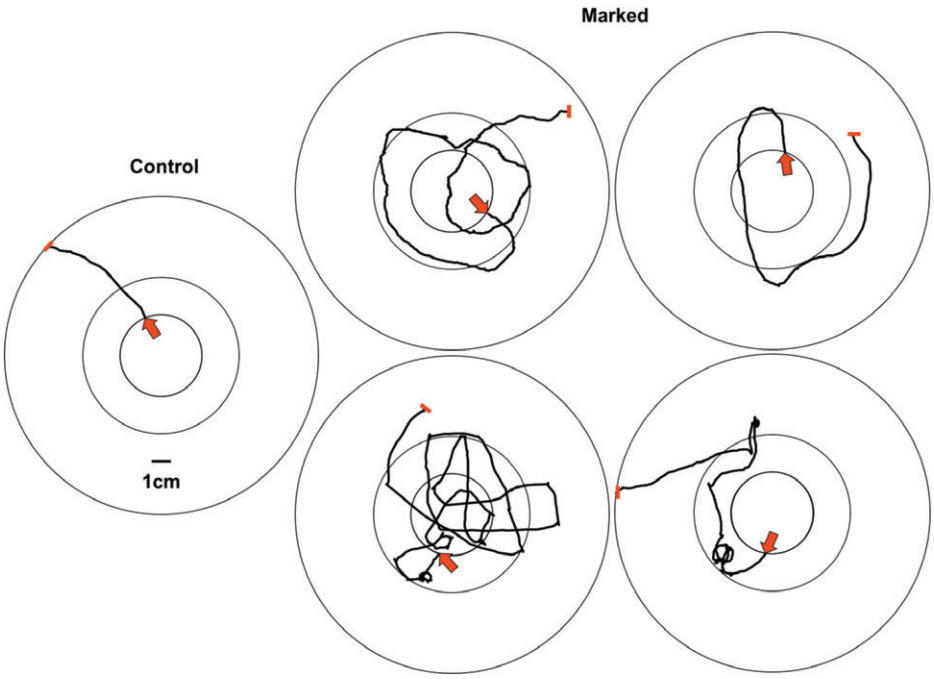


Fig. 2. Tracks of five *Lucilia sericata* larvae during different attractive signal experiments (one Control and four Marked trials). The apparatus was comprised of three zones: a Centre Zone (the circle in the center), a Marked Zone (represented by the ring; marked by 20 starved larvae for 30 min; for Control trials, this zone was unmarked) and an Outer Zone. The start point of each bioassay (trial) is represented by the arrow and the end-point by the small line. The trajectory pattern that was observed in the Control trials was the same for all of the other trials.

Study of the scanning behaviour

This experiment was designed to measure the scanning response according to larval signal. During scanning, the larva stopped and moved the anterior part of its body back and forth laterally (Green *et al.*, 1983). The experimental set-up was a circular arena of diameter 9 cm as in Boulay *et al.* (2013). Test larvae were isolated in wet pine-wood dust for 30 min prior to the experiments to remove any traces of their former breeding substrate (beef liver). A disc of clean paper towel covering the whole surface of the Petri dish was first humidified homogenously with 1.5 mL of distilled water. The 'Control' (C) consisted of no deposit on this paper (only the presence of distilled water). The 'Food' condition (F) consisted of 200 µL of a solution containing 3 g of mixed beef liver diluted in 50 mL of distilled water. The solution was deposited using a micropipette such that the food solution was spread over the entire arena. The 'Larvae' condition (L) consisted of the signal created by 10 larvae moving freely within the arena for 10 min and removed before the start of the trial. These signal-depositing larvae were previously starved and cleaned for 4 h in wet pine-wood dust (time sufficient to obtain larvae with empty crops Charabidze *et al.*, 2013) to eliminate food odours acquired from the breeding substrate (Boulay *et al.*, 2013). The 'Larvae + Food' condition (L + F) consisted of the deposition in the arena of both the larval signal and the food substrate (in that order). Twenty replicates were performed for each condition and individuals were followed for 5 min. Behavioural observations were video recorded using a digital camera (Dinion LTC0355; Bosch, Germany) and analyzed with ETHOVISION, version 8.0 XT (Noldus). Mean absolute meander/tortuosity, which is defined as the change in direction of movement of an individual relative to the distance (i.e. the amount of turning per unit distance) was used to describe the changes in larval orientation (Granchietti *et al.*, 2012) [absolute meander = (turn angle/distance moved); Noldus *et al.*, 2001]. The encoding of scanning behaviour was made from the video recording using *sequenceR* (a free interface developed by M. Hervé and available online; http://www.maximeherve.com/r-statistiques/sequencer) implemented in R software (R Development Core Team, 2008). The number of scanning motions was noted for each experimental condition.

Larval path visualizations were obtained by leaving individuals free to move on wet ground with no deposit for a few minutes. The paths were photographed using a smartphone (IPhone 5C, 8 Mpx; Apple Inc., Cupertino, California) and edited using PHOTOSHOP CC, version 14.2 (Adobe Systems, San Jose, California). The images illustrated clearly the environmental exploration of each individual larva.

Statistical analysis

Because the attractive signal experimental data were not normally distributed, nonparametric statistical tests (Mann–Whitney) were used to compare mean time and mean distance travelled in each zone by larvae under Control and Marked conditions. Then, bilateral tests ($U_{\alpha} = 1.96$ for d.f. = 1; if

$U > U_{\alpha}$, it is considered to be significant) were used to compare the proportion of time spent in each zone (CZ, MZ and OZ) between Control and Marked conditions.

Also, because directional movement and scanning behaviour data were not normally distributed, nonparametric tests (Kruskall–Wallis, Dunn and Mann–Whitney) were used to compare larvae trajectories under all conditions and to test the effect of substrates on scanning behaviour (Zar, 2010). All tests were performed using INSTAT, version 3.06 (GraphPad Software Inc., San Diego, California). The level of significance α was set at 0.05.

Results

Set-up analysis

The present study was designed with regard to previous work from this laboratory focusing on the larval signal (Boulay *et al.*, 2013), as well as personal observations of larval scanning behaviour. First, the signal-following ability of larvae (larval trail experiment) was studied. Then, to test the hypothesis that scanning behaviour may be involved in such signal detection, the nature and quantification of scanning behaviour were explored under three relevant (natural) conditions and a blank control.

The larval instars used during the present study were young third-instars. With this type of instar, sensory responses as previously observed did not differ from those of second-instars (Cobb, 1999), and their size (length) was sufficiently large to permit tracking of larvae using trajectometry software (ETHOVISION, version 8.0 XT; Noldus). Individual larvae were followed under infrared wavelengths, thus avoiding any visible light stress (Hinnemann *et al.*, 2010). Larvae were cleaned using wet pine-wood as in our previous (Boulay *et al.*, 2013). This method could be inefficient in removing all food traces but has been tested using larval signals on one half versus food on the other half. In this experiment, larvae spent significantly more time in the food zone (Boulay *et al.*, 2013). In another binary choice experiment between food + larval signal versus food, individuals are observed to stay preferentially in the food + signal zone (J. Boulay, unpublished observations). These results demonstrate the existence of the 'larval signal' exists, and that its retentive effect differs from signals based on food only. Accordingly, the cleaning method that was used to remove food traces from the cuticles of larvae appears to be effective.

The proportion of scanning movements observed during 5 min of recording decreased under all four conditions between the first minute (mean ± SD; Control: 0.3 ± 0.25; Food: 0.33 ± 0.27; Larvae: 0.21 ± 0.17; Larvae + Food: 0.21 ± 0.23) and the last minute (Control: 0.11 ± 0.16; Food: 0.08 ± 0.11; Larvae: 0.17 ± 0.18; Larvae + Food: 0.14 ± 0.19). Thus, a decision was made to analyze scanning behaviour only during the first minute of the experiments because, from previous observations and mean larval velocity, this first minute was sufficient for larvae to explore the entire arena (Charabidze *et al.*, 2008; Boulay *et al.*, 2013).

Larval trail experiment

Larval signals had a retentive effect. When conspecific larvae had been formerly present in the set-up, test larvae spent significantly more time in the marked zone than those under the Control condition (mean ± SD; Control: 16.1 ± 13 s; Marked: 108.9 ± 125 s; U = 29, $P < 0.0001$). Larval trajectories under the Control condition were in straight lines with no about-turns or visible foraging (Fig. 2). Accordingly, the mean distance travelled by individuals in the experimental set-up was shorter under the Control condition than under the Marked condition (mean ± SD; Control: 6 ± 3 cm; Marked: 22.4 ± 21 s; $U = 30$, $P < 0.0001$). Furthermore, in the absence of a signal (i.e. the Control condition), larvae never returned to the CZ; they crawled straight away from centre to the outside of the arena (Fig. 2). On the other hand, larvae placed in the set-up marked by larval signal clearly detected the MZ and stayed preferentially within it (Fig. 2). Under these conditions, the larval trajectories were more sinuous and many larvae were able to follow the boundaries of the MZ (Fig. 2). From a more quantitative point of view, the proportion of time spent in CZ and MZ under the Marked condition was significantly higher than under the Control condition (Marked versus Control: for CZ, $U = 7.58$, $P < 0.001$; for MZ, $u = 14.14$, $P < 0.001$) (Fig. 3). Under the Control condition assays, larvae spent significantly more time in the Outer Zone (OZ) than during the Marked condition assays (for OZ, $U = 27.98$, $P < 0.001$) (Fig. 3).

Study of the scanning behaviour

The absolute meander was significantly higher under Signal conditions (Larvae, Food and Larvae + Food conditions) than under Control conditions, meaning that larvae had trajectories that were more tortuous when a signal was present in the arena (KW = 34.5, $P < 0.001$). No significant differences were observed for the absolute meander between Signal conditions (Dunn's tests: L versus F: $P > 0.05$; L versus L + F: $P > 0.05$; F versus L + F: $P > 0.05$).

The mean number of scanning movements varied according to test signals. Larvae scanned significantly more under the Control and Food conditions than under Larvae and Larvae + Food conditions (C versus L: $U = 81.5$ $P < 0.01$; C versus L + F: $U = 74$, $P < 0.001$; F versus L: $U = 121.5$, $P < 0.05$; F versus L + F: $U = 83$, $P < 0.01$) (Fig. 4A). No significant difference was observed between the Control and Food conditions (C versus F: $U = 163.5$, $P > 0.1$) (Fig. 4A) and Larvae and Larvae + Food conditions (L versus L + F: $U = 142.5$, $P > 0.1$) (Fig. 4A), again showing that scanning was decreased in the presence of the larval signal.

The larval signal also increased the time between two successive scannings: this time was significantly higher under Larvae and Larvae + Food conditions than under Control and Food conditions (C versus L: $U = 1341.5$, $P < 0.05$; C versus L + F: $U = 900.5$, $P < 0.01$; F versus L: $U = 1086$, $P < 0.05$; F versus L + F: $U = 728.8$, $P < 0.01$) (Fig. 4B). Regarding the number of scans, the time between two successive scannings did not differ between Control and Food conditions ($U = 2832.5$, $P > 0.1$)

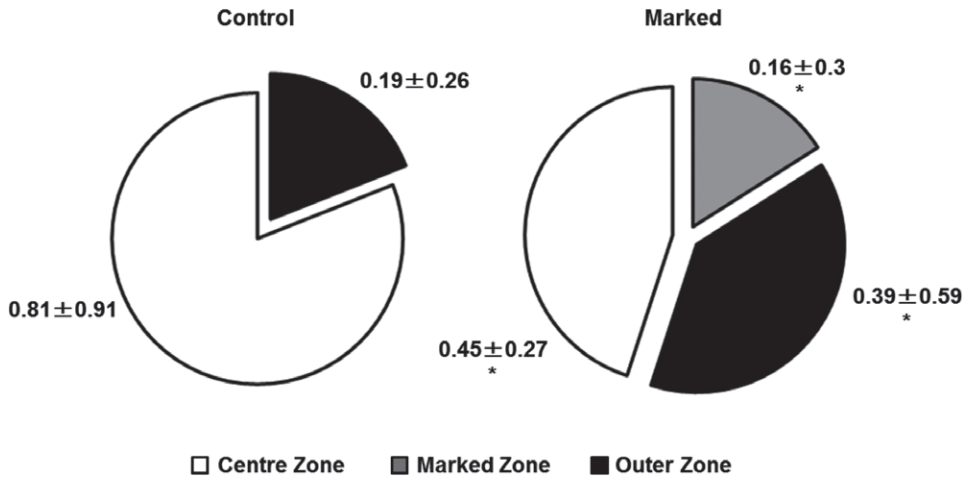


Fig. 3. Mean ± SD proportion of time ($n = 20$) spent by *Lucilia sericata* larvae in each of the behavioural trial zones. Asterisks (*) show significant differences between Control and Marked trials for each of the zones using bilateral tests ($U_\alpha = 1.96$ for d.f. = 1).

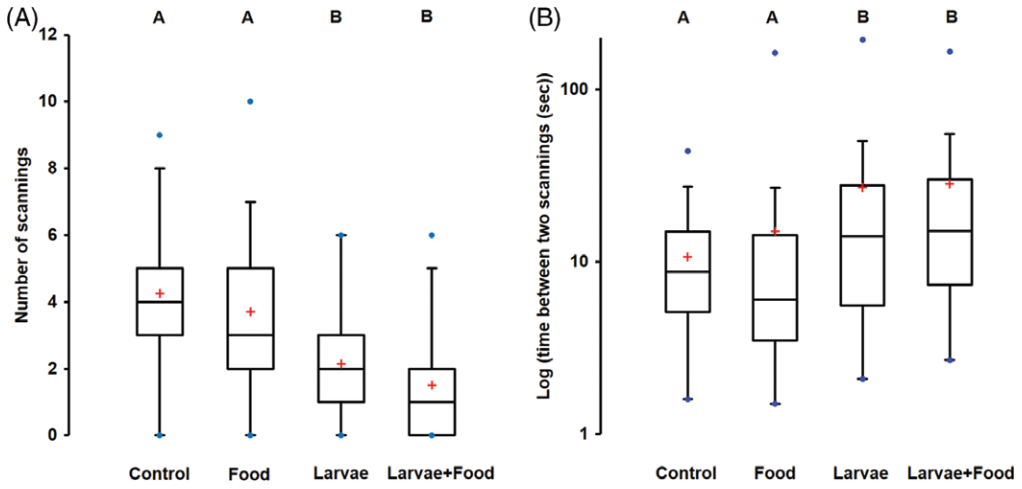


Fig. 4. (A) Boxplots of number of scanning behaviours performed by *Lucilia sericata* larvae under each of the experimental conditions during the first minute of the experiment ($n = 20$; uppercase letters show significant differences in Mann–Whitney tests). (B) Boxplots of time (s) between two scannings of *L. sericata* larvae under each of the experimental conditions during the first minute of experiment ($n = 20$; uppercase letters show significant differences in Mann–Whitney tests). Crosses represent the means.

(Fig. 4B), nor between Larvae and Larvae + Food conditions ($U = 585.5$, $P > 0.1$) (Fig. 4B).

The larval path clearly showed the places where individuals scanned (Fig. 5). During their exploratory behavioural movements, larvae have intertwined locomotion and scanning (Fig. 5).

Discussion

Study limitations

The authors are aware that the present study does not definitely confirm the implication of larval signal and scanning in the aggregation of the presocial larval blow fly. However, from the results obtained, it is reasonable to assume that the trail-following abilities of the larvae and the retentive effect of the 'larval signal' are involved in the aggregation process. Furthermore, although the exact role of scanning is not confirmed, there is a clear link with the presence of larval signal, strongly implying a role in the aggregation behaviour.

Larval trail

The present study is designed to analyze the detection of a conspecific signals (as yet unidentified) by the necrophagous larvae of the Green bottle blow fly *L. sericata* and to explore their signal-following abilities. First, the results demonstrate that *L. sericata* larvae are able to detect the boundaries of a signal left by conspecifics and their preference to stay within the prescribed area. In the absence of signal from conspecifics,

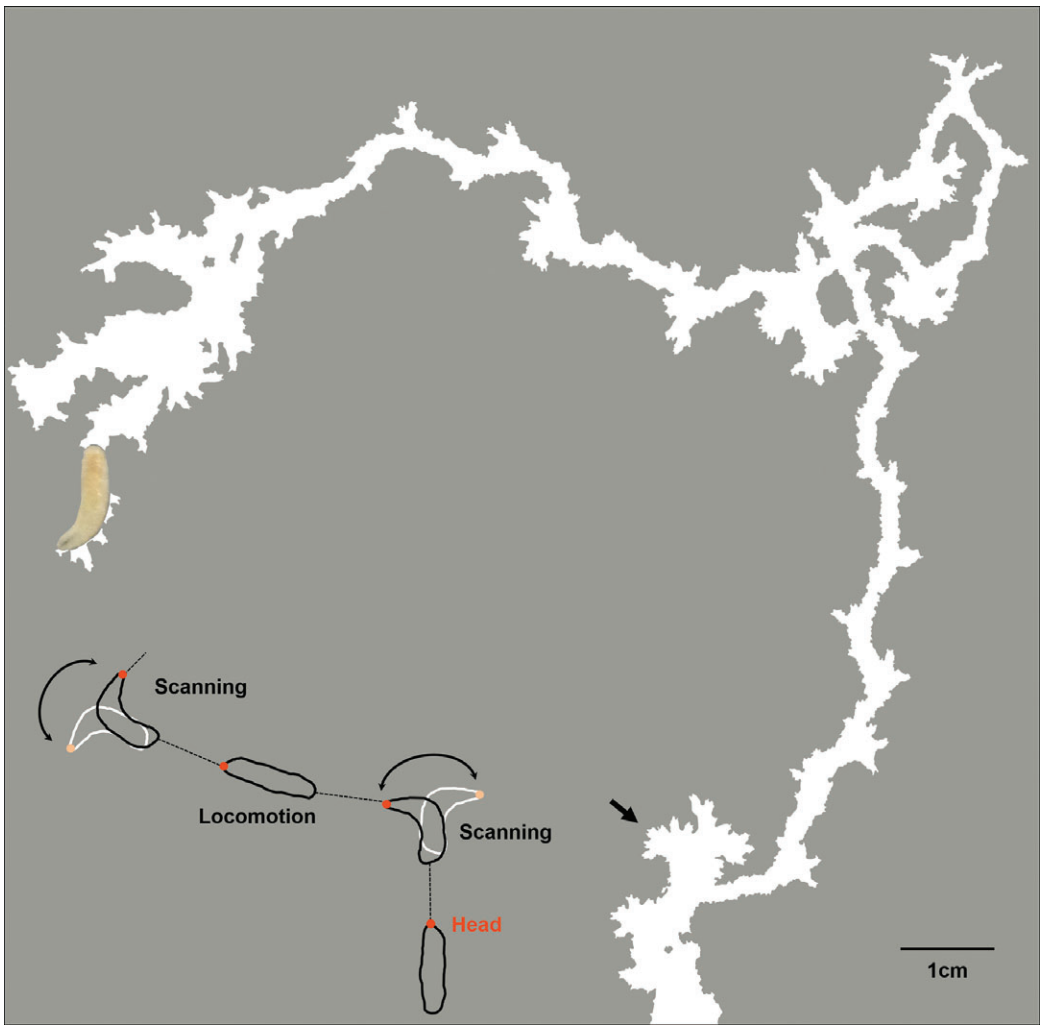


Fig. 5. A scheme of locomotion and scanning behaviour of *Lucilia sericata* larva. During scanning, also named *bending* (Green *et al.*, 1983), the larva is at the stop and successively turns the anterior third of its body in right and left directions. After few seconds scanning is over, the larva moves in the chosen direction. The path of one *L. sericata* larva in a new environment is illustrated in white. The illustration (modified from a photograph) shows the area of physical contact between the larva and the substrate. The black arrow indicates one place where scanning occurred, with successive head positions depicting a tree-like structure. The larva is visible at the left.

larval trajectories are straight with no about-turns. However, the prior presence of conspecifics (i.e. larval signal) impacts on the larval behaviour (Fig. 2). Their trajectories are more sinuous, with numerous turns: larvae explore the arena instead of moving out of it (Fig. 2). Accordingly, the *L. sericata* larvae are able to detect a signal formerly left by conspecifics and alter their locomotion accordingly. A similar behaviour, based on biased running (i.e. very sinuous trajectories), is observed during chemotaxis in *Drosophila* larvae (Gomez-Marin *et al.*, 2011; Gomez-Marin & Louis, 2012; Ohashi *et al.*, 2014). This strategy is confirmed by regarding the branching tree-like path of *L. sericata* larvae during an environment exploration (Fig. 5). Larvae scan at each stop, turn slightly and continue their movement. The larvae are acquiring information about their environment (e.g. chemical gradient detection) by scanning, and are then moving accordingly.

Scanning behaviour

The results show that *L. sericata* larvae scan similarly under Control and Food conditions, although significantly less when a larval signal is present (Fig. 4B). Under both Control and Food conditions, the larval signal is absent: under these conditions, the larvae scan many times, and their successive scannings are close in time to each other. Such a scanning pattern is visible in Fig. 5, which illustrates the result of numerous scans and changes in orientation. This finding agrees with that of Green *et al.* (1983), who report no difference in the scanning frequency (called 'bending' in their study) of larval *Drosophila melanogaster* between the food and no-food environments. On the other hand, individuals tested under Larvae and Larvae + Food conditions in the present study are directly in contact with the larval signal. They scan for less time and less often. Accordingly, it

is reasonable to assume that, in *L. sericata*, larval scanning is involved in the detection of the chemical signal deposited by conspecifics on or in the substrate (i.e. klinotaxis: Fraenkel & Gunn, 1961; Gomez-Marin & Louis, 2012). Further experiments will be required to identify the chemical composition of the larval signal (e.g. using gas chromatography; Roux *et al.*, 2008), as well as the involvement of the compounds identified in conspecific recognition (using behavioural tests).

Aggregation of blow fly larvae is maintained by an active behaviour that is likely supported by a larval signal (Boulay *et al.*, 2013). The detection of conspecific cues (tactile and chemical) is essential for larvae to be able to (re)join the group and to benefit from aggregation advantages (Rivers *et al.*, 2011). The results reported in the present study suggest that larvae use the scanning behaviour not only for turning, but also to compare ground signals on either side (klinotaxis) and to orientate accordingly (Fraenkel & Gunn, 1961). Larval masses are self-organized systems. One of the mechanisms of such social organization is the additive action of a signal that permits amplification of the system (Camazine *et al*., 2001). According to the results of the present study, it is reasonable to conclude that scanning could be used by individual *L. sericata* larvae to follow the trails left by conspecifics and/or to locate the most crowded areas. However, a precise description of the sensory organs of *L. sericata* larvae and a demonstration of a direct linkage between these structures and the larval signal chemodetection are still needed. Such a linkage is already reported for larvae of other dipteran species, such as *Musca domestica* (Chu & Axtell, 1971a,b), *Drosophila melanogaster* (Oppliger *et al.*, 2000) and *Hylemia* sp. (Honda & Ishikawa, 1987).

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