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Original Article

The maggot, the ethologist and the forensic entomologist: Sociality and thermoregulation in necrophagous larvae

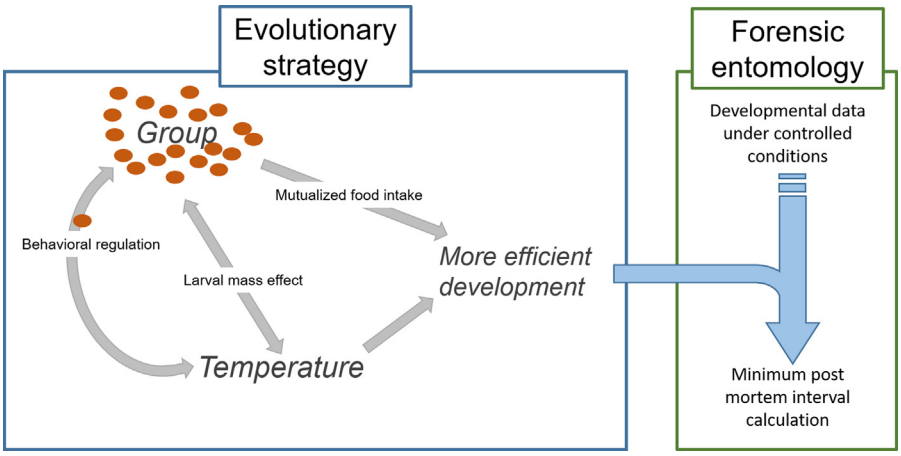
Cindy Aubernon *, Valery Hedouin, Damien Charabidze

Univ-Lille, CHU Lille, EA7367-UTML-Unite de Taphonomie Medico-Legale, F-59000 Lille, France

HIGHLIGHTS

- Necrophagous blowflies larvae maintain a permanent balance between thermal regulation and aggregation.
- These two parameters affect their development.
- Such a behavioral regulation likely optimize their development on carcasses.
- This may be a pre-social strategy to cope with harsh environment.
- Forensic entomology studies should consider the behavior of maggots.

GRAPHICAL ABSTRACT



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ABSTRACT

Necrophagous insects are mostly known through forensic entomology. Indeed, experimental data investigating the effect of temperature on larval development underlies post-mortem interval estimations. However, such developmental studies rarely considered the behavior of maggots. In contrast, previous results supposed that calliphoridae larvae use behavioral strategies to optimize their development on carcasses. To test this idea, we analyzed the trade-off between thermal regulation (individual thermal preferences) and social behavior (aggregation) in *Lucilia sericata* larvae. The first set of experiments analyzed the behavior of third instars in response to thermal changes in their environment. The results demonstrated a clear thermoregulation behavior, supporting the assumption that larvae continuously move to reach a suitable internal temperature. The second set of experiments focused on the trade-off between thermal optimization and aggregation. The results showed a constant search for congeners and an attractiveness of aggregates, sometimes to the detriment of thermal optimization. Together, these results demonstrate a balance between behavioral thermoregulation and social strategies, two significant mechanisms for developmental optimization in necrophagous larvae. In conclusion, these findings highlights unexpected (social) strategies to cope with ephemeral resource and high selection pressure. They also raise important questions for forensic entomology.

Introduction

Aggregation, often considered as the first stage of sociality, can be described as a simple inter-attractive behavior resulting in a

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* Corresponding author.
E-mail addresses: cindy.aubernon@gmail.com, cindy.aubernon@univ-lille.fr (C. Aubernon).

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local increase in individual density (i.e., an aggregate) [1]. This behavior can be observed during larval and/or adult life stages and be composed of individuals of a single or sometimes different species [2]. In any case, aggregation is based on direct or environmentally-mediated (i.e., stigmergy) communication and involves feedback loops [3,4]. While apparently simple, such stochastic and repeated interactions between members of a group can result in complex structures and serve critical roles [5].

To understand the developmental strategies of such group-living animals, reductionist approaches (i.e., focusing on individuals) are limited. Indeed, the study of single individuals placed under strictly controlled conditions noticeably fails to explain how larger scales of organization influence the behavior and *in fine* the fitness of these individuals. A more realistic consideration is that complex systems have features that none of their individual parts have and must be studied as a whole. As an example of this approach, Dombrovski et al. recently discovered cooperative behavior in *Drosophila* larvae [6]. While foraging in liquid food, larvae aligned themselves and coordinated their movements to drag a common air cavity and access deeper food. According to the authors, this social cooperation could be a strategy to cope with a harsh environment. As insects breeding on fresh carcasses face high selection pressures, they provide interesting opportunities to study such social adaptations. As with *Drosophila* larvae [6], blowfly larvae may have developed complex social strategies resulting in better development on carrion.

From a niche-partitioning point of view, blowflies (Diptera: Calliphoridae) can be regarded as a pioneer species; they are the first colonizers of vertebrate carcasses [7]. Calliphorid larvae (i.e., maggots) are among the few insects able to grow on fresh necromass (i.e., animal's carcasses), and they dominate the carrion ecosystem during the first decomposition stages [8]. Their growth is strongly correlated with heat: in a range of favorable conditions, larval development speed increases linearly with temperature [9]. Due to its importance for calculating the minimum post mortem interval (mPMI) [10], this relationship between temperature and blowfly larval development has been extensively studied in the context of forensic entomology. While the first studies on maggot development time [11] focused on the effect of ambient temperature, later research has shown that behavior also affects larval development [12]. A striking example is the larval mass effect [13,14]. This local heat emission is the consequence of larval crowding and can increase local temperatures above 40 °C, resulting in faster development of aggregated larvae [15,16]. These results shed light on the impact of social strategies on larval development and the limitations of development data based on reductionist experiments. In the present study, the hypothesis is that individual and social thermal regulation behavior may exists in blowflies necrophagous larvae.

At the individual level, most ectotherms regulate body temperature using microhabitat selection [17,18]. Compliant with this idea, necrophagous larvae have been observed to adapt their foraging activity according to local temperature [19]. Larvae are also able to move toward a thermal gradient to locate and select a preferred species-specific temperature [20]. The authors hypothesized this temperature as a trade-off allowing larvae to grow fast but efficiently (i.e. large individuals with low mortality rate). Furthermore, Scanvion et al. [12] demonstrated that aggregation facilitates exodigestion and food intake, thus contributing to a shorter development time and better fitness of aggregated larvae. Accordingly, a trade-off between individual (thermal regulation) and social (aggregation) behavior may exist. To test this idea, the present work analyze the trade-off between thermal regulation (individual thermal preferences) and social behavior (aggregation) in calliphoridae larvae.

Material and methods

Insect breeding

Lucilia sericata adult flies (approximately 250 ± 50) were reared in 50 × 50 × 50 cm tulle cages with caster sugar and water *ad libitum*. Eggs were obtained by placing a pillbox of 20 ± 1 g of mixed beef liver inside the insectarium during a maximum of four hours. Presence of eggs was checked hourly: laying time was thus known with a more or less 30-minute resolution. The eggs obtained were kept in closed plastic boxes (143 × 105 × 59 mm) inside a climatic chamber (Sanyo, Moriguchi City, Osaka, Japan) at 19 ± 0.1 °C on 100 ± 5 g of mixed beef liver until reaching the appropriate instar [9].

Thermal regulation behavior

The experimental setup named *choice setup* consisted of a 40 × 5 × 5 cm gutter-like metallic bar containing 250 ± 5 g of mixed beef liver. This bar was closed with an opaque plastic lid and kept at 21 ± 2 °C ambient temperature. Tow heating pads (Groupe Thermo Technologies, Annecy, France, Schutzart IPX4) placed at each extremity under the bar created two hot spots (HS). iButton thermometers were deposited every 5 cm (from 2.5 to 37.5 cm) inside the liver to monitor local temperatures (DS1921G Thermochron iButton, accuracy: 0.5 °C; Maxim Integrated, San Jose, CA, USA).

The same protocol was used for all experiments; only the temperature of the hot spots and durations changed. Eighty third instars were removed from rearing boxes and placed in a pillbox to starve [21]. After 4 h, these larvae were spread over the bar, one each half centimeter, and the bar was closed. At the end of the experiment, the lid was opened, and the bar was divided into four 10 cm sections. The larvae in each section were counted, and the temperature was recorded.

Four different experiments were performed using this setup. (A) The ability of larvae to select and aggregate on a hot spot was analyzed (*A-Single hot spot*). For this purpose, only one spot was heated at 27 °C, while the rest of the bar was at ambient temperature (Fig. 1A). The location of larvae was analyzed after 8 or 16 h with 17 and 18 replicates respectively. (B) The ability of larvae to locate and select the warmest spot was investigated (*B-Two hot spots*). For this purpose, one spot was heated at 27 °C, while the second spot was set at 36 °C (Fig. 1B). This last temperature is close to that one observed by Aubernon et al. [20] as the preferential value for this species. Two durations, 8 and 16 h, were investigated (15 replicates). (C) The ability of aggregated larvae to relocate on a hot spot (27 °C) when the temperature of their local environment (36 °C) decreased was analyzed (*C-Hot spot cooling*). This experiment thus mimics temperature changes on a carcass during night time (surface temperature drop). For this purpose, the experiments started with two hot spots turned on: one at 27 °C and the second at 36 °C. After 16 h, the 36 °C spot was turned off while the other spot stayed at 27 °C for eight more hours (14 replicates). (D) Finally, the ability of aggregated larvae to move to a new and hotter spot (*D-Hotter spot*) was investigated. For this experiment, a first spot was heated to 27 °C. After 8 or 16 h, a second spot was turned on at 36 °C for 16 or 8 h, respectively, so that the total experiment duration was always equal to 24 h. Sixteen replications were performed for each condition.

Aggregation vs. thermal optimization

To analyze the trade-off between aggregation and thermal optimization, larvae were placed in a thermal gradient

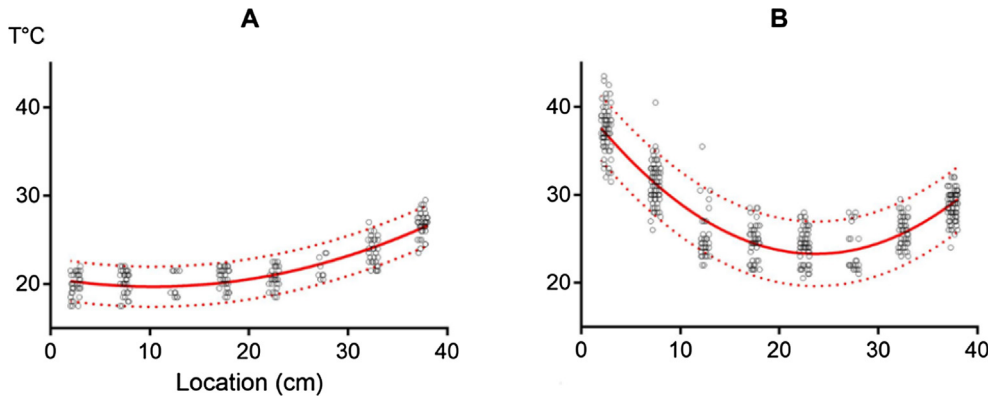

Fig. 1. Curves representing the temperature inside the choice setup (each 5 cm) at the end of the experiment. Dotted lines represent the boundary markers for 2.5 and 97.5 percentiles. A: Thermal profile when only the less warm spot is turned on. B: Thermal profile when the two hot spots are turned on.

(Thermograde, [20]) with a conspecific captive group located at a sub-optimal temperature. In brief, Thermograde is composed of a heating shelf and a gutter-like galvanized steel bar (80 × 5 × 5 cm). Before each experiment, 500 ± 5 g of fresh mixed beef liver was spread inside the bar to create a 2 cm high food layer. The heating shelf underneath the bar created a linear thermal gradient inside the beef liver ranging from 22 ± 0.5 to 49 ± 0.5 °C (i.e., a 0.36 °C increase every cm). Forty third instars were homogeneously spread on the setup. A captive aggregate formed by 40 or 20 third instars enclosed in a tulle bag (5 × 5 × 0.2 cm) with 10 pieces of polyethylene foam (0.1 ± 0.01 g) was placed in the colder area of the Thermograde. After 19 h, the number of larvae located inside each 5 cm section was counted (14 replicates with 40 larvae in the tulle bag, and 8 replicates with 20 larvae). A control experiment was performed using the same setup but an empty tulle bag (7 replicates).

Statistical analysis

For part A to D, the insect count (i.e., presence or absence) in each area have been modelled using a logistic regression under a quasi-binomial distribution assumption. Binary choices between areas for one replicate have been analyzed using z-tests. For part E, a dendrogram based on a hierarchical clustering approach was created to qualify the differences between replications (experiments with a ratio of 20/40). Finally, Mann-Whitney test has been used to compare mean temperature selection to the one reported in Aubernon et al., [20]. Logistic regression and hierarchical clustering have been performed using R software v.3.3.2 (R development Core Team). Z and Mann-Whitney tests were performed using XLStat (XLStat, Addinsoft, Paris, France, 2016).

Results

The experiments were performed on a natural food substrate (ground beef liver), in the dark, and at realistic larval densities. Due to this experimental design and the burrowing behavior of larvae, it was not possible to monitor individuals in real time. To prevent any disturbance of larvae, their location in the setup have been observed only once per trial at the end of the given experimental time (8, 16, 19 or 24 h). In other words, the results observed after 16 h were not the pursuit of 8 h experiments, but a second set of experiments lasting longer. While these methods are more time consuming than repeated monitoring of the same experiment over time, it allows observation of the exact location of all the larvae without disrupting aggregates or exposing larvae to light and other stress factors. Using this setup, the mean survival rate for all our experiments was 91.53 ± 7.44%.

Thermal regulation behavior

Single hot spot

The thermal gradient inside the choice setup was shaped as a curved slope with the base at 20.19 ± 0.74 °C and the top at 26.66 ± 0.32 °C (Fig. 1A). Under these conditions, larvae promptly moved inside the bar and gathered on the hottest spot. Results clearly shown the majority of the larvae in the warmer area: 98.59 ± 1.18% of the larvae after 8 h and 98.69 ± 2.70% after 16 h (Fig. 2). No difference was observed between these two durations (logistic regression: estimate = −0.35, p = 0.635).

Two hot spots

The thermal gradient inside the choice setup was bowl-shaped, with one side at 27.33 ± 1.27 °C and the other side at 37.32 ± 1.29 °C, while the central area was at 24.53 ± 3.58 °C (Fig. 1B). After 8 h, 71.06 ± 19.98% of individuals were located on the hottest spot, and one third were observed on the other side (27.33 ± 1.27 °C, Fig. 3A). However, after 16 h, the repartition shifted with 95.16 ± 3.19% of individuals located on the warmer

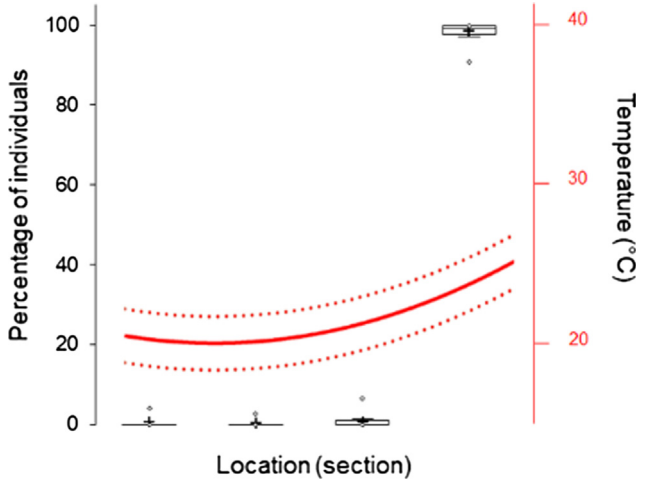

Fig. 2. Chart representing the location of the larvae according the temperature inside the choice setup with a single 6.66 ± 0.32 °C hot spot turned on. The solid red line represents the temperature (°C), and the red dotted lines represent the boundary markers for 2.5 and 97.5 percentiles. Box plots represent the percentage of maggots in each of the four sections of the choice setup. The horizontal line inside the box represents the median, the cross the mean, and the dots represent minimum and maximum. The lower and upper limits of the box are the first and third quartiles, respectively. Whiskers indicate the 1.5 × interquartile range.

spot (Fig. 3B). Statistical analyses showed that larval repartition differed according to experiment duration: significantly more larvae were located on the warmer spot after 16 h than after 8 h (logistic regression: estimate = 2.08, $p = 1.61e^{-08}$).

Hot spot cooling

After 16 h on, the hottest spot (37.32 ± 1.29 °C) was turned off, resulting in a cooling of the spot and a shift in temperature profile (from Fig. 1B to Fig. 1A). The results showed that 8 h after this shift occurred, 94.17 ± 8.10% of the larvae moved from this first spot and relocated to the 27.33 ± 1.27 °C spot (Fig. 3C). This distribution significantly differed from that observed when the 2 spots were turned on (logistic regression: estimate = −7.001, $p < 2e^{-16}$).

Hotter spot

Larvae were located on the 27 °C spot during the first 8 or 16 h (see A- Single Hot Spot). When the 36 °C spot was then turned on (for 16 h or 8 h, respectively), a slight displacement of the larvae toward this warmer spot occurred (for 16 h logistic regression estimate = 4.65, $p = 2.47e^{-4}$; for 8 h logistic regression estimate = 5.17, p = 0.001). Considering each experiment by itself (Fig. 4), it was observed, for most replications, a unimodal repartition with a significant choice for one of the two spots (z-tests for all replicates: $z > |2.68|$, $p < 0.03$). However, for six replicates out of 48, the repartition was not different from a 50/50 one (z-tests for all 6 replicates: $z < |1.78|$, $p > 0.06$).

Aggregation vs. thermal optimization

Placing a bag containing 40 captive larvae at 23 ± 1 °C resulted in 98.72 ± 2.80% of the 40 free larvae moving at 23 ± 1 °C (Fig. 5). Using 20 captive individuals, which is half as many as free individuals, the choice was not as strict, with a non-homogeneous aggregation between replicates (cf. cluster dendrogram on Fig. 5). However, larvae were always located between their preferential temperature and the captive larvae. On the opposite, control experiments showed that an empty bag did not affect the location of the larvae inside the Thermograde (compared to Aubernon et al. [20], Mann-Whitney test: U = 41, p = 0.45).

Discussion

Necrophagous larvae are mostly known from forensic entomology research [11]. Most of the developmental data have been obtained in this context, focusing on the effect of ambient temperature on development time [9]. Not surprisingly, the majority of these studies have been performed under similar conditions, using constant and homogeneous temperatures, easy to ingest food and a restricted number of insects [9,22]. Furthermore, the experimental procedure often includes regular measurement or sampling, and thus, the perturbation of aggregated larvae [23]. Analyzing larval behavior was not an issue; Grassberger and Reiter specifically designed their Material and Methods to "achieved a more two-dimensional and disseminated feeding behavior, which is essential to prevent maggot mass formation" [9]. Moreover, parameters that determine population fitness (e.g., survival rate) were not studied [24]. However, there is a growing recognition that several biotic parameters, and more particularly behavior, affect larval development and fitness [25]. The present study highlight complex behavioral strategies likely resulting in a better development on carrions, and suggest how

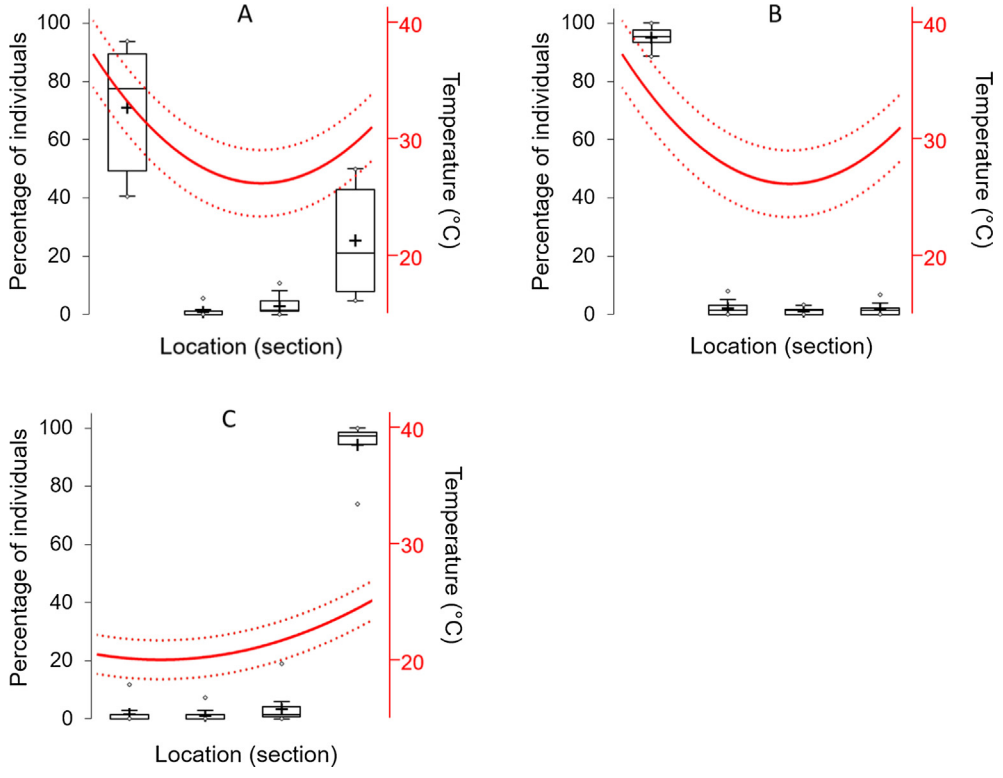

Fig. 3. Chart representing the location of the larvae according the temperature inside the choice setup. The solid red line represents the temperature (°C), and the red dotted lines represent the boundary markers for 2.5 and 97.5 percentiles. Box plots represent the percentage of maggots in each of the four sections. The horizontal line inside the box represents the median, the cross the mean, and the dots represent minimum and maximum. The lower and upper limits of the box are the first and third quartiles, respectively. Whiskers indicate the 1.5 × interquartile range. A and B: Representation when the two spots are turned on during 8 and 16 h, respectively. C: Representation at 24 h when the warmer spot had been turned off for 8 h.

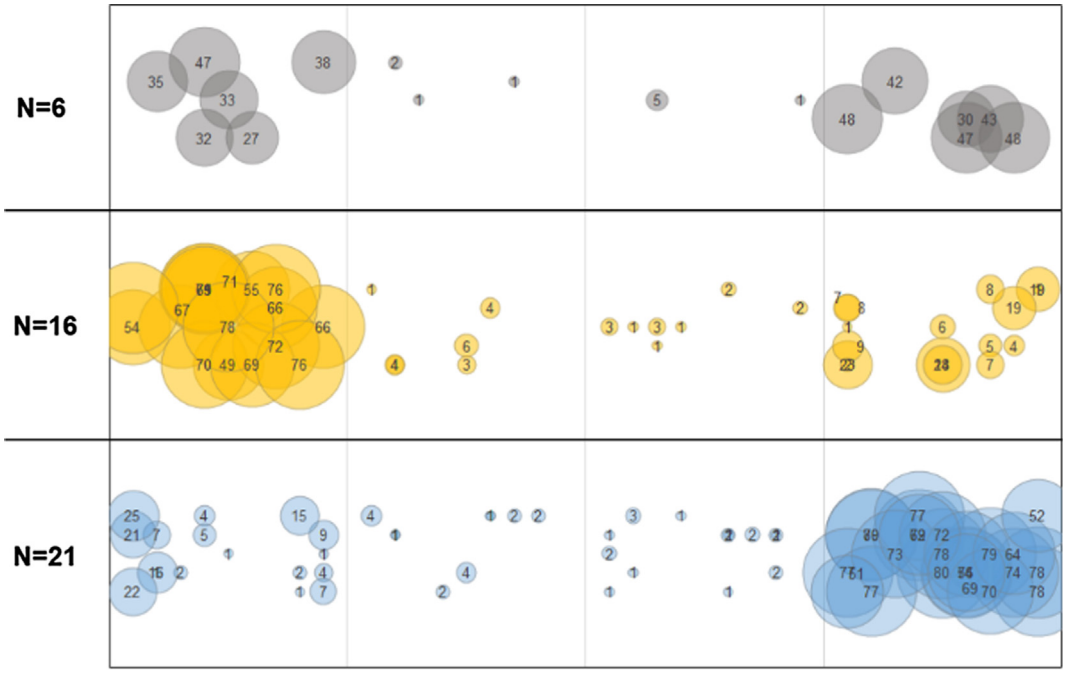

Fig. 4. Repartition of larvae inside the apparatus according to their choice to move to the warmer spot or to stay in their previous aggregation area. Bubbles represent the percentage of larvae per each replicate. In gray, at the top, is representation of the 6 replicates without choice. In yellow, at the middle, is representation of 16 replicates when larvae moved to the warmest spot. In blue, at the bottom, is representation of 21 replicates when larvae stayed on the less warm spot (i.e. their initial place of aggregation).

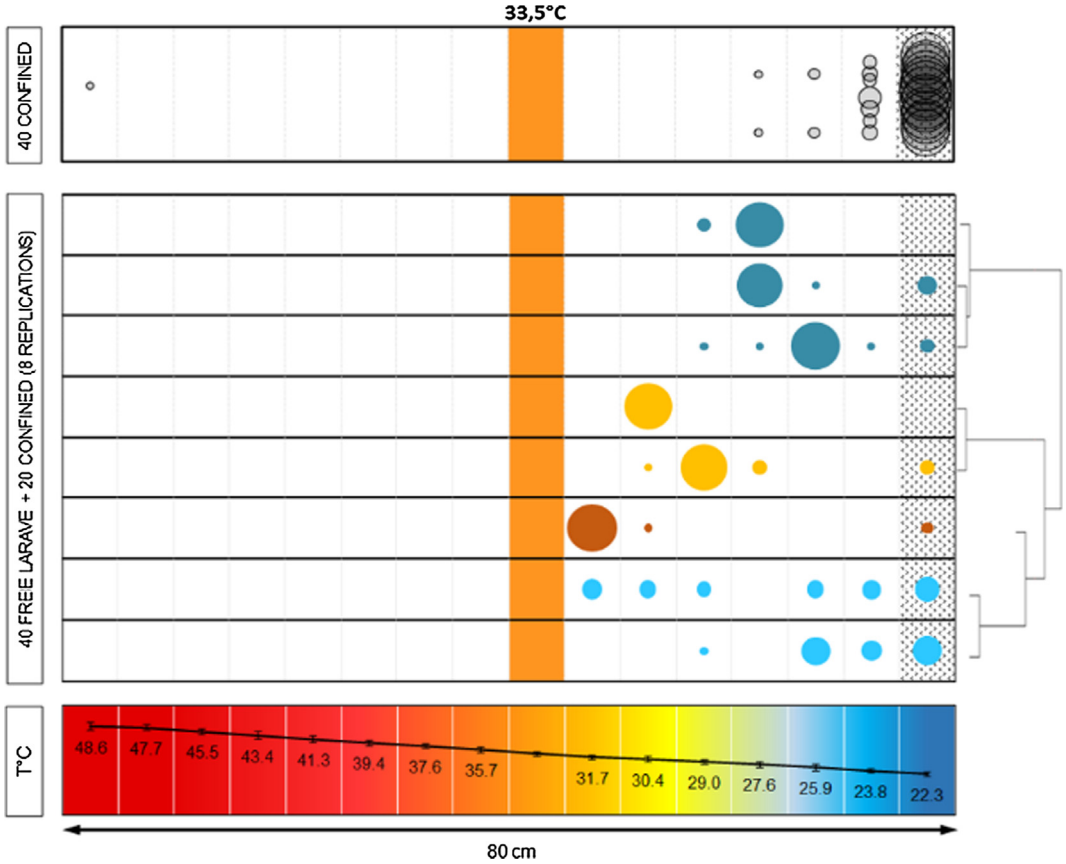

Fig. 5. Representation of the location of larvae inside the Thermograde experiments. At the bottom, the colorful scale describes the temperature sample in the Thermograde from 22.3 to 48.6 °C. The orange bar identifies the location of the preferential temperature of *L. sericata* and thus the selected area in previous experiments. At the top, the bar represents all the replicates when 40 free larvae confronted 40 trapped maggots. In the middle, each bar represents one replicate when 40 free larvae confronted 20 trapped maggots. These replicates are compared using the dendrogram placed on the right of the chart.

forensic entomology could benefit from this behavioral ecology point of view.

Thermoregulation

Heat is especially important for the development of insect larvae. The local temperature prevailing during larval development noticeably impacts their survival rate [26], activity [27], development speed [9] and morphometrics [28], as well as adult size and fertility [29,30]. Thus, temperature is a key parameter not only for larval growth but also for the fitness and other life history traits of the population [24,29]. Consequently, most ectotherms regulate body temperature to some extent using behavioral mechanisms such as changes in posture and microhabitat selection [17,18]. As necromass is a rich but ephemeral resource with high selection pressure, it has been shown that necrophagous larvae should tend to grow fast, which can be obtained by favoring high local temperatures [9]. To test this idea, an observation of the reaction of larvae to temperature changes has been made. Results demonstrate a thermal regulation behavior and supports the assumption that larvae continuously move to maintain a suitable internal temperature [19,31,32].

Results demonstrated that larvae were able to locate hot spots and preferentially aggregated on these areas. Whether with one or two hot spots turned on, larvae gathered on the hotter place. When only one hot spot was present, this aggregation occurred directly; when two hot spots were available, aggregation was achieved in two steps. Larvae initially moved to a near hot spot, resulting in two different aggregates; in a second step, all larvae gathered on the hotter spot. This unbalanced proportion of larvae on the two spots during the first step, with the two thirds on the hotter spot and the other third on the less warm spot, strongly suggests a gradient-following behavior. Indeed, the inflexion point of the thermal curve occurred on the third part of the setup (Fig. 1B). It is thus likely that the larvae first followed the ascending gradient on their side resulting in the 2/3 VS 1/3 initial repartition. Compliant with this result, larvae already aggregated on a hot spot (less warm spot) also reacted to the warming of a distant location (warmer spot). In contrast, when a location became less favorable (cooling of the warmer spot), larvae moved to the other hot area that was previously avoided. These experiments demonstrate that larvae not only search for high temperatures, but also for the best available temperature.

In calliphorids larvae, both development and growth rate increase with temperature, and the balance of the two also determine how adult size changes [9,29]. Although fast growth seems to be generally favored by natural selection, it also carries costs, and individuals grow more often at a lower rate than they are physiologically capable of [35]. Thus, growth results in a trade-off between development speed and quality (as defined in [33;34], without taking into account the reproductive performance). Aubernon et al., [20] demonstrated that *L. sericata* larvae placed in a thermal gradient selected a 33.3 ± 1.52 °C area to aggregate. According to the authors, this choice could be the optimum allowing larvae to optimize both development duration and quality. While this temperature is quite high (in most European countries, such temperatures are only punctually recorded under field conditions), local temperatures inside large larval masses often reach or exceed this threshold [13,15].

Social behavior

Calliphorids larvae are also known for their gregariousness, resulting in large maggot-masses gathering hundreds to thousands of individuals [31]. This social behavior brings several advantages in terms of fitness [31]. Accordingly, we hypothesized a trade-off between individual (thermal preference) and social (aggregation) behavior to optimize larval development.

Compliant with this idea, this study highlight an effect of the group on the selected temperature. In other words, there was a retentive effect of the group, and this retention prevented larvae from relocating to a more suitable temperature. During experiments involving the warming of a second spot, larvae initially aggregated on the first spot (less warm) did not always move to the later hotter spot (Fig. 4). It is supposed that the benefits of aggregation and the cost of moving might have balanced the benefits of a higher local temperature. Here, the benefits of aggregation could be a mutualized food intake (i.e. exodigestion) that permits a nourishment facility [12]. Consistent with this idea, Padmanabha et al. [36] observed that Aedes aegypti larval development is sensitive to the combination of nutrient and thermal conditions. It is important to note here that in any case, a group of 40 larvae does not produce heat; the larval mass effect has only been demonstrated for larger groups gathering hundreds to thousands of larvae [37]. Furthermore, such a retention cannot be simply explained by group inertia; indeed, aggregated larvae were observed moving during cooling experiments. Thus, a more complex balance between aggregation and thermoregulation must be involved.

In a second set of experiments, larvae faced a choice between aggregation and thermal optimization. In such conditions, they always selected the 40-larvae group located at cold temperatures (23.52 ± 0.81 °C) rather than the uncrowded but hotter area. Such a result confirm the existence of a trade-off between aggregation and thermal optimization. Interestingly, the same experiment performed with a group of only 20 captive larvae resulted in more qualified results. Instead of gathering with the fewer larvae located in the cold area, the 40 free larvae were found spread between 33 ± 0.5 °C and 24 ± 0.5 °C or aggregated at an intermediate temperature (i.e., 26.82 ± 2.54 to 29.68 ± 2.39 °C). Larvae were therefore able to assess the number of aggregated larvae and/or the costs/benefits of joining the group, and adjusted their behavior accordingly. Compliant with this idea, Fouché et al. [38], demonstrated that blowfly larvae can discriminate the signals of different species and to infer the quantity of larvae from ground-deposited cues.

Conclusions

From an adaptive point of view, gregariousness is often considered a strategy to cope with harsh environments, particularly through protection against predation and parasites [39]. Additional specific benefits have also been demonstrated for necrophagous larvae, namely, collective exodigestion and heat emission [12,13,31]. Overall, the reason for a given larva to stay within an aggregate appears to be a balanced choice considering at least some immediate costs (displacement, cold temperature) and benefits (high temperature, collective exodigestion, protection against predators and parasites). To conclude, larval behavior appears to be a complex trade-off between the search for congeners (i.e., aggregation) and suitable environmental conditions, particularly, an optimal local temperature. Due to the rarity of high (i.e., close to 34 °C) ambient temperature in central Europe, aggregation can thus be regarded as an alternative way to reduce development time through mutualized food intake [12] and the emergence of social phenomena such as larval mass effects [13].

From a more practical point of view, these findings should be kept in mind when performing developmental studies or caseworks in forensic entomology. Since maggot's behavior and group retention strongly affect the temperature experienced by larvae, we can suppose that development time could be impacted as well.

However, given the complexity of behavioral regulations, it appears utopian to establish *a posteriori* the exact temperature experienced by larvae during their development. Consequently, mPMI calculation errors might appear, particularly in cases with high temperature variations. Therefore, we recommend increasing the margin of error on the development time calculations, especially in cases involving strong thermal variations and weak larval density. We also suggest reconsidering the way forensic entomology development data are obtained to include the social behavior of larvae in forthcoming studies.

Conflict of interest

The authors have declared no conflict of interest.

Compliance with Ethics Requirements

All institutional and national guidelines for the care and use of animals were followed.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2018.12.001.

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