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Effect of various odour attractants on egg-laying activity of black soldier flies (*Hermetia illucens*)

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Abstract

The black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae), is a cosmopolitan fly. Its feeding behaviour, high productivity and the high value of its larvae in macronutrients (mainly lipids and proteins) makes this species one of the promising candidates for large scale insect farming and organic waste management. The reproductive rate, female oviposition preference and oviposition attractants of *H. illucens* are understudied. A better knowledge of the attractants involved in oviposition site choice would help maximising black soldier fly reproductive success. The current study aims to compare the efficacy of four food attractants on the oviposition activity of *H. illucens* females. The trial was conducted in a cage (11 × 1.25 × 0.9 m) with a volume of 12.38 m^3 and containing 83,610 adults (6750 adults/m^3). The cages were placed in a rearing room where the conditions were: T = 28 ± 1 °C, RH = 70 ± 5%, photoperiod of 16 h light: 8 h dark and light intensity = 8140 Lux). The tested feeds included a rabbit feed, fish waste (sardine, sardinella, and anchovy waste), a mixture of tropical fruits (pineapple, banana, papaya and mango), and fresh cattle manure. The performance of each feed attractant was evaluated in terms of the mass of eggs laid on the laying medium. This study showed that fruit mixture is the most efficient attractant, followed by fresh cattle manure and fish waste while the rabbit feed was the least efficient. The results of this study can be used to optimise the reproduction of the black soldier fly.

Keywords

food attractants – insect feed – mass rearing – oviposition preference – reproduction

1 Introduction

With a growing world population that will reach nine billion people by 2050 (Alexandratos and Bruinsma, 2012), and increasingly demanding consumers, global food production will need to produce enough food for the population while combatting its negative environmental impacts, such as increased greenhouse gas emissions, monoculture, biodiversity loss, and unsustainable water use (Foley *et al.*, 2011; Gerten *et al.*, 2020) which

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will add pressure on increasingly scarce agricultural resources and ecosystems (Van Huis *et al.*, 2013).

One approach to address this is to use insects as a new ecologically sustainable food source (Hermans *et al.*, 2021). Insects have a high food conversion potential compared to other homeothermic animals that are losing more energy to regulate their body temperature. Insects are poikilothermic and show less energetic losses (Pinotti and Ottoboni, 2021). They emit much less greenhouse gases than conventional livestock and also have rich and balanced nutritional composition making them suitable for human and animal nutrition (Van Huis and Tomberlin, 2017). Black soldier fly (BSF; *H. illucens*, Diptera: Stratiomyidae) larvae have attracted the attention of the scientific and entrepreneurial community for their potential to provide promising solutions to two growing global problems, namely livestock feed production and organic waste management through bioconversion (Bosch *et al.*, 2020; Miranda *et al.* 2020). BSF larvae are capable of consuming twice their body weight per day (Hermans *et al.*, 2021). They consume a wide range of organic residues such as agricultural coproducts and organic wastes of plant and animal origin (Kim *et al.*, 2021). In addition, BSF larvae represent a protein and lipid rich biomass that can be used as for animal feed (Moula *et al.*, 2018; Randazzo *et al.*, 2021; Van Huis *et al.*, 2013) or biodiesel production (Wang *et al.*, 2017). In order to make this solution environmentally friendly, the development of efficient rearing and breeding methods on an industrial scale are necessary (Cičkova *et al.*, 2015; Gao *et al.*, 2021). The production of large quantities of good quality eggs is one of the key steps in the industrial rearing of this species in order to ensure rearing continuity while recycling large volumes of organic matter (Hoc *et al.*, 2019; Pastor *et al.*, 2015).

During artificial breeding of BSF, adult flies are maintained in a mating cage to provide continuous supply of eggs. When upscaling production, it is important that females lay eggs at the same spot to facilitate egg collection. To do so insect farms usually use attractants associated with the eggies (sites of oviposition).

In the wild, females lay eggs in dry crevices above or around moist decaying organic matter (Tomberlin *et al.*, 2002; Zheng *et al.*, 2013) that will be later used as food source for their larvae. Female attractiveness for egg-laying is thus based on the smell of fermented organic matter. Manure (Boafo *et al.*, 2023; Gougbedji *et al.*, 2021), decaying meat or fish (Bonso, 2013) are thought to provide conditions that meet the reproductive needs of adult females. However other sources such coffee grounds or sweet potatoes can also be used as efficient attractants (Romano *et al.*, 2020). Although several studies have already tried defining the best attractant for females, no result have so far been conclusive.

The objective of the present study is to compare the attractiveness of different food attractants to the BSF females, while giving the insect the choice to select the most appropriate oviposition site, in an artificial rearing system. The attractants used in this study are: fresh cow manure, fish waste, moistened rabbit feed, and a mixture of fermented fruits (pineapple, banana, papaya and mango). The performance of each attractant was evaluated in terms of the mass of eggs laid on each oviposition medium.

2 Materials and method

Experimental site

The experimentation was carried out in a reproduction chamber at the laboratory of applied entomology of the Agronomic and Veterinary Institute HASSAN II horticultural complex of Agadir (Morocco). We used a mating cage (11 m width, 0.9 m length and 1.25 m height) covered with green net (mesh size: 2 × 2 mm) for the experiment. The climatic conditions of the rearing chamber were controlled and kept at 28 ± 1 T°C and 70 ± 5% RH. The cage was equipped with 8 medium and 4 large openings to facilitate manipulations inside the cage. Eight 80 W white LED lamps were used at the top of the cage to maintain a photoperiod of 16 h light: 8 h dark, and one other at a light intensity of approximately 8140 Lux (110 PPFD (µ mol/m^2/s). Four taro root plants (*Colocasia esculenta*) and four Mexican fan palms (*Washingtonia robusta*) were put inside the greenhouse to serve as support for the BSF adults. Four water containers were disposed throughout the cage to provide unlimited water supply (Figures 1 and 2).

Biological material

The adult black soldier flies (*Hermetia illucens*) used in this experiment came from the mass rearing maintained in the laboratory of entomology of the horticultural complex of Agadir (IAV-CHA). They were reared on the waste of the university restaurant in plastic containers (37 × 31 × 10 cm). The environmental conditions of the breeding are maintained in total darkness at 29 ± 1 °C and 70 ± 5% RH. To have homogeneous pre-pupae, 18 days old larvae were put in a very moist substrate (80% water content) to force mature larvae to leave the substrate into a pre-pupae collection system. After 24 hours of harvesting, manual sorting was done to keep only pre-

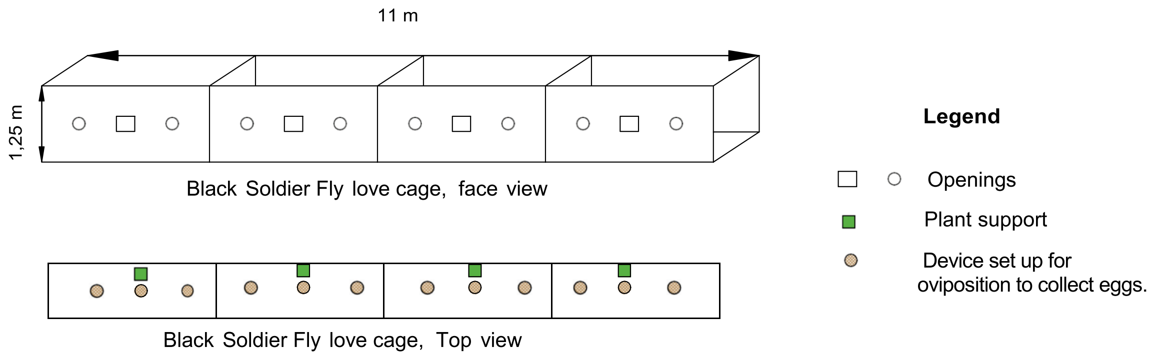

FIGURE 1 Autocad assisted drawing of the *Hermetia illucens* adult rearing greenhouse.

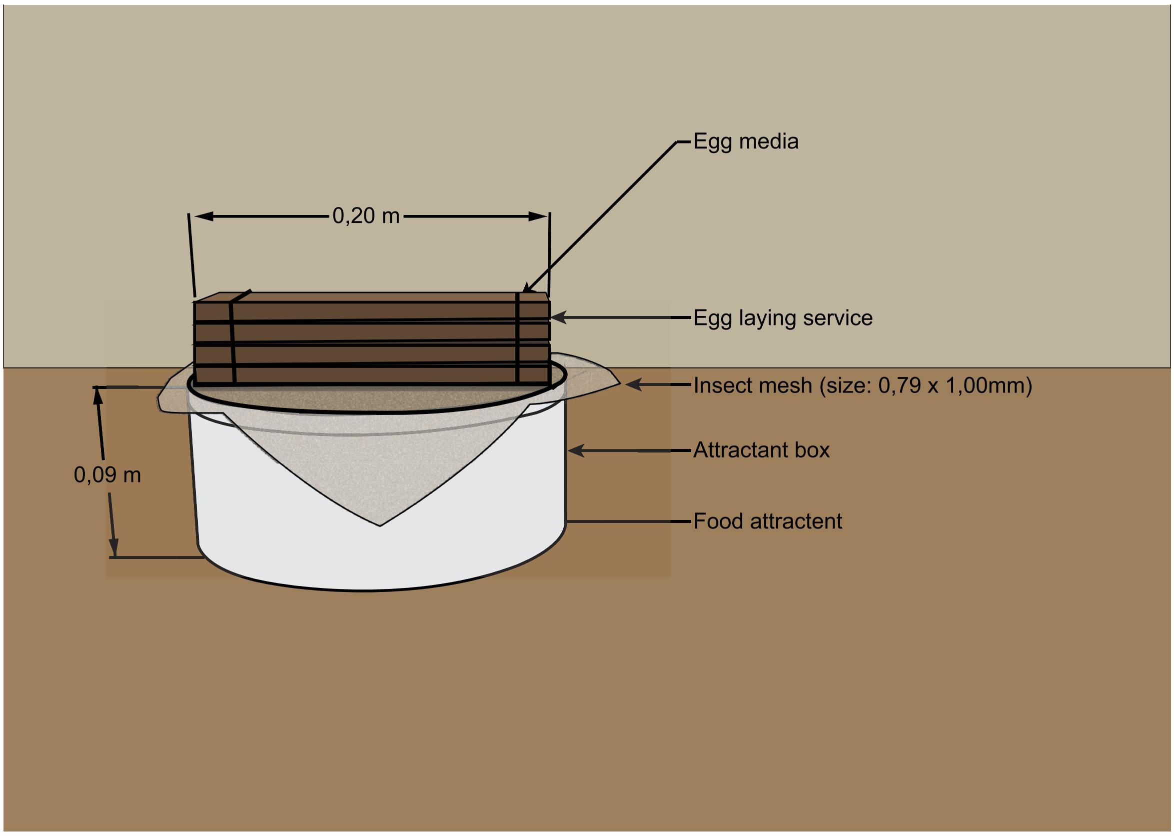

FIGURE 2 Oviposition device set up for egg collection.

pupae (brown coloured larvae). Approximately 92,000 pre-pupae (equivalent of a 7500 ind./m^3, Hoc *et al.*, 2019), were put directly into the love cage, while keeping the breeding chamber in the dark until the emergence of first adults after 14 days.

Odour attractants

Four odorant baits were used in the study: cattle manure, fruit mixture (pineapple, banana, mango, papaya and avocado), rabbit feed composed of corn, barley, oats, alfalfa, wheat and soybean meal and a mineral vitamin compound, manufactured by ALF ISSEN (Taroudant, Morocco), and fish waste (mixture of sardine, sardinella, and anchovy waste).

Preparation of the spawning tanks

The fresh odorant baits (300 g) were placed in a cylindrical plastic container (22 cm diameter, 9 cm height) and covered with a nylon anti-thrips net (mesh size: 0.15 × 0.15 mm) to prevent flies from laying eggs on the net or directly in the food, or to prevent flies from falling and drawing into the feed while ensuring the diffusion of odours in the cage. The attractants were prepared 2 days before experiment to ensure minimum fermentation, and were replaced every 4 days to avoid fungal contamination.

Experimental setup

Due to the homogeneous conditions of the mating cage, the experimental design adopted was a completely randomised design. Three repetitions per bait were made, with a distance of 83 cm between hangers.

Monitoring and collection of eggs

Adults usually start to mate two days after emergence, and females start to lay eggs two days after mating (Tomberlin and Sheppard, 2002). We thus started to collect eggs four days after first flies emerged. The wooden eggies containing the laid eggs were removed and gently scraped with a stainless-steel knife. The eggs were weighed using an analytical balance (SAB 32, 0.1 mg, bay SCALTEC (Labexchange, Burladingen, Germany)). This operation was repeated every day until most adults were dead. Afterwards, the collected eggs were placed on a fine plastic mesh (2 × 2 mm) positioned over a plastic container filled with larval feed. The food used for this purpose is chick starter feed, consisting of proteins (20.0%), cellulose (3.2%), minerals (5.5%), fats (4.5%), calcium (0.9%) and phosphorus (0.6%). The ratio employed was 60 g of wet food (with 75% water content) for every 1 g of eggs. The plastic containers were then placed in the incubation chamber controlled (27 ± 1 °C, 65 ± 5% RH, total darkness).

Adult emergence

The rate and dynamics of adult emergence were also assessed to quantify the number of adults in the mating cage. Twenty random samples of 96 pupae were monitored. Emptied pupae were counted every day and removed from each box. Non-emerged flies were considered abnormal or dead (Figure 3).

Reproductive parameters

The reproductive parameters evaluated in this study were the following:

$$\text{Adult emergence rate (\%)} = \text{number of adults emerging} / 96(\text{number of pupae used}) \times 100 \quad (1)$$

$$\text{Egg weight per egg media} = \text{total weight of eggs laid} \quad (2)$$

$$\text{Pre-oviposition period} = \text{time between first emergence and first egg laying} \quad (3)$$

$$\text{Egg laying period} = \text{time between the first and last egg laying} \quad (4)$$

Statistical analysis

The statistical analyses were performed using R software (R Development Core Team, 2022). Data were analysed using ANOVA and generalised linear model (GLM, R function *glm*) with a Gaussian error distribution. Post hoc pairwise comparisons were made using Tukey's HSD test (R function *glht* from the R package multcomp; Hothorn *et al.*, 2008).

3 Results

Adult emergence

The cumulative emergence curve showed that on average 90% of the flies emerged (Figure 4) with the highest rate of emergence (33%) occurring on the third day after the start of the trial. Using these data, we can consider that a total of approximately 85,500 flies emerged (6970 ind./m^3) (Figure 5).

emergence rate per day and grey dots represent all emergence data points (20 samples).

Reproductive parameters monitored

The mating period began on the second day after adult emergence and continued until the 8th day, during which adult flies exhibited a declining trend in mating. The pre-oviposition period lasted for two days. The observed oviposition period spanned ten days, with the highest oviposition rate occurring on day six following the initial oviposition (Figure 5).

Effect of food attractants

Although all baits were to some extent attractive to BSF females, the type of odourant bait used had a significant impact on the total weight of eggs laid per eggy (GLM, Df = 3, $P < 0.001$; Figure 6). Fruit mixture showed the highest attractiveness; however, it was not significantly higher than fish waste ($Z = 2.12$, $P = 0.14$; Figure 6). Fruit mixture showed however higher attractiveness than cattle manure ($Z = -3.12$, $P = 0.009$; Figure 6) and rabbit feed ($Z = 5.28$, $P < 0.001$; Figure 6). Fish waste showed higher attractiveness than rabbit feed only ($Z = 3.16$, $P = 0.008$; Figure 6) and wasn't more attractive to BSF egg-laying females that cattle manure ($Z = -1$, $P = 0.074$; Figure 6). No difference in the amount of eggs laid in eggies exposed to rabbit feed odours compared to eggies exposed to cattle manure odours ($Z = 2.1$, $P = 0.13$; Figure 6).

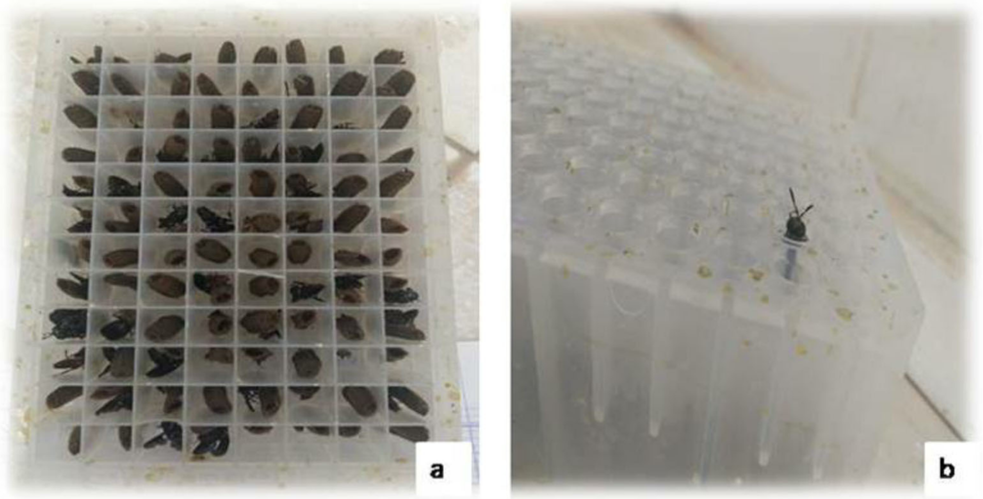

FIGURE 3 Evaluation of the emergence rate of *Hermetia illucens* adults. (a) observation box containing pupae (n = 96); (b) emergence of an adult.

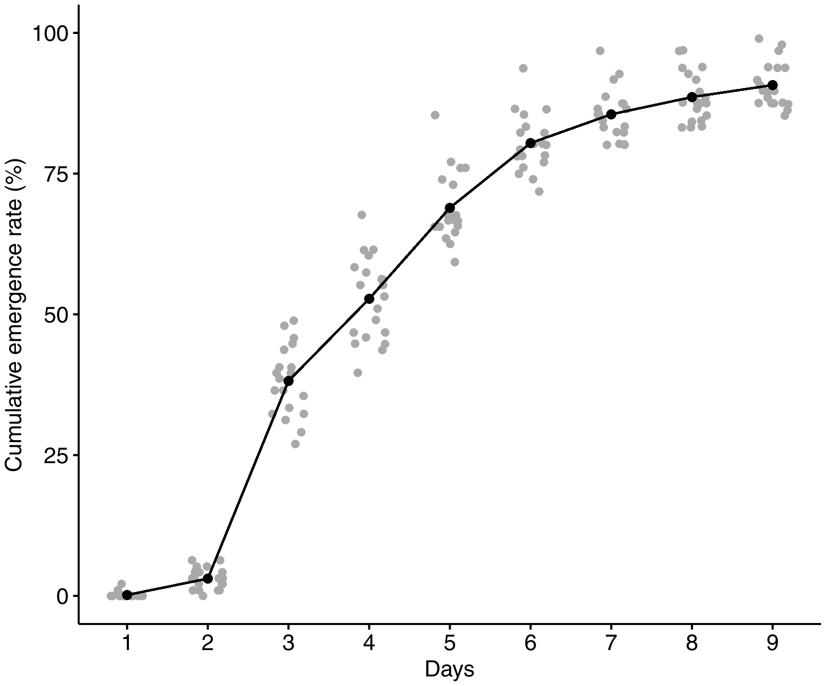

FIGURE 4 Cumulative emergence rates of black soldier fly adults. Blacks dots represent average emergence rate per day and grey dots represent all emergence data points (20 samples).

4 Discussion

Reproductive parameters

The oviposition period recorded in our study (9 days) is pretty similar than those reported by Nakamura *et al.*, (2016) showing an oviposition period ranging from 7.6 ± 0.8 to 9.4 ± 0.8 days at 25 °C. Oonincx *et al.* (2016) however observed a slightly longer oviposition period (10.0 ± 3.5 at 26 °C) whereas Zhang *et al.* (2010) observed a much longer one (16.0 ± 5.3 days at 26 °C). The slight difference with Nakamura *et al.* (2016) could be explained by the adult selection method used. The authors manually selected the adults emerging simultaneously for their experiments while in our experiment individuals emerged randomly thus increasing observed heterogeneity in developmental times and lengthening adult emergence period. Our method was chosen considering the ideal number of individuals needed per m^3 to maximise reproduction and to reflect the reality of mass rearing conditions. The peak of emergence recorded in day three is in accordance with other studies showing highest emergence four to eight days after

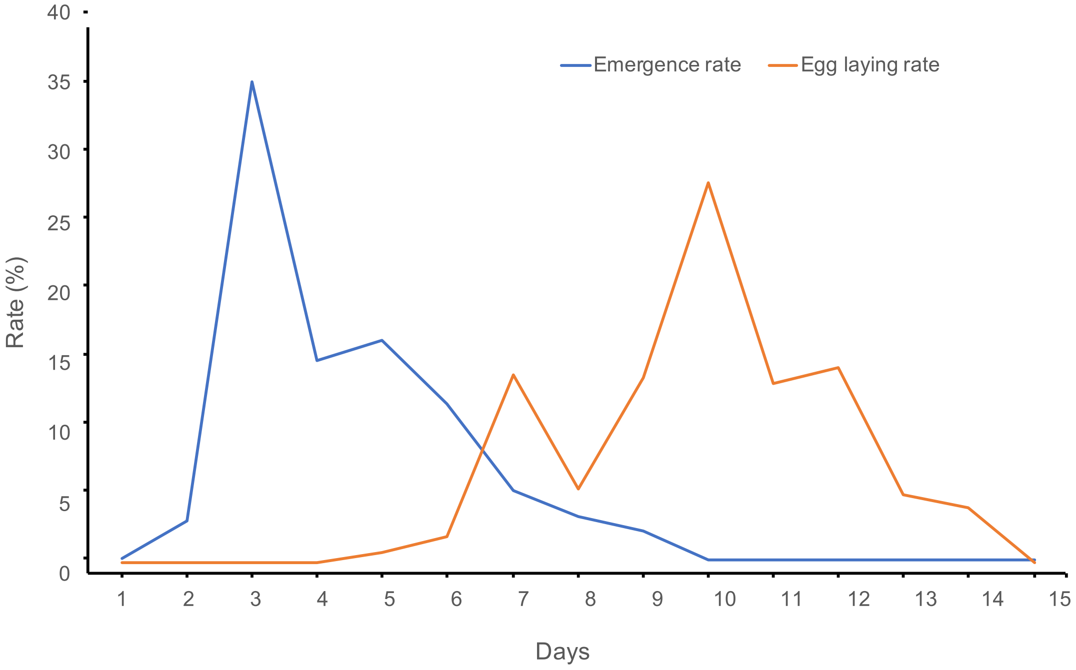

FIGURE 5 Adult emergence rate (blue line) associated with egg laying rate (orange line) of black soldier fly females per day.

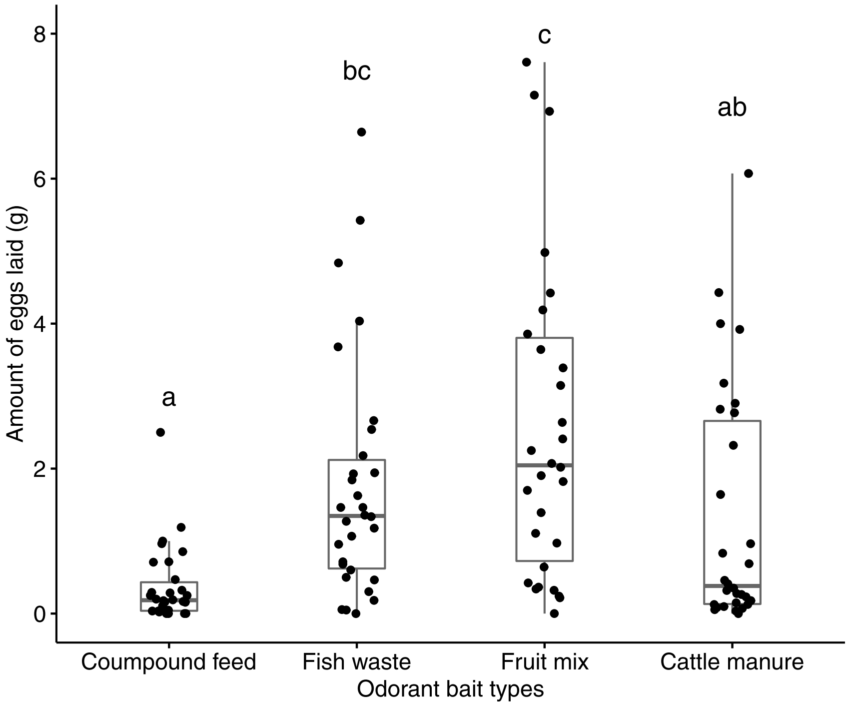

FIGURE 6 Total weight of eggs laid per oviposition site (n = 3 × 10 days) associated with the various odourant bait types. Box plots show the median (black line) and 25-75% percentiles. Whiskers show all data. Black dots represent all data points. Letters indicate significant differences between treatments (GLM, Tukey's HSD test, ($P < 0.05$).

first flies emerged (Heussler *et al.*, 2018; Nakamura *et al.*, 2016).

Hoc *et al.* (2019) observed the highest egg production at the highest adult density tested of 8500 adults/m³, however they didn't try higher densities. Cičkova *et al.* (2012) estimated, based on extrapolated data from common houseflies (*Musca domestica*) that 13,000 ind./m³ might result in optimal egg production. In our experiment the population density was almost twice less than potential optimal density so higher amount of eggs laid and oviposition duration.

Adult emergence rate

The peak of emergence recorded on day 3 is 36%, it is a bit earlier but at a comparable rate compared to the peak observed by Caruso *et al.* (2014), which is 40% in the seventh day after pupal transfer to the insectarium.

The effect of food attractants

Black soldier flies are generalist saprophagous insects, they feed on a wide range of decaying organic material from fruits and vegetables to animal manures and cadavers (Lord *et al.*, 1994). For generalist species, the selection of a suitable food source for their offspring is critical as the quality of food substrates has a huge impact on larval fitness. They may have to locate and assess the quality of many different potential decaying substrates in their environment before one is accepted for oviposition. To do so, females use olfactory stimuli to locate oviposition sites (Gouinguené and Stadler, 2006). The volatile organic compounds (VOCs) constituting these stimuli are generally included in a mixture of odours emanating from different sources in the insect's environment (Bruce *et al.*, 2005). They are perceived by insects through a highly sensitive olfactory system that allows them to accurately locate a suitable oviposition site.

In nature, BSF adults are usually found in areas with high amounts of organic matter (Tomberlin and Sheppard, 2001). Studies have shown that decaying food emits volatile compounds into the air that are detected by mated BSF females (Sripontan *et al.*, 2017). Bonso (2013) tested the attractiveness of BSF females to different decaying substrates (fish waste, chicken feed, human faeces and rat meat carcasses), and showed that rat meat and human faeces induced higher attraction compared to fish waste and chicken feed. This shows that egg-laying females seem to have innate preferences for certain substrates over others.

Our results indicate that the fruit mixture was the most attractive for BSF females and stimulated more oviposition activity compared to rabbit feed and cattle manure, however not significantly more than fish waste. This finding is in accordance with another study reporting that fruit odours was more effective in attracting black soldier flies (Sripontan *et al.*, 2017). However, this does not mean that BSF females have an innate higher preference for fruits, because oviposition preference of dipteran insects may be affected by previous exposure to foods. Information from earlier feeding experiences is known to be exploited by generalist insects to facilitate accurate decision-making when selecting an oviposition site (Lhomme *et al.*, 2018, 2020). This phenomenon is called natal habitat preference induction (NHPI, Davis and Stamps, 2004) and has already been demonstrated in generalist saprophagous insects including fruit flies (*Drosophila melanogaster*) and house flies (*M. domestica*) (Lihoreau, 2016; Ray, 1999). Original diet used to rear BSF consisted of food waste, including mostly vegetables and fruits, thus we can't exclude a potential experience-based preference induction for fruits by BSF females.

In natural conditions, oviposition site selection by BSF females might also be based on its nutritional value. Other saprophagous insects such as fruit fly females are known to use gustatory stimuli to assess the quality of oviposition sites (Yang, 2008). However, in this experiment BSF females didn't have direct access to the substrates and were therefore not able to assess substrate quality using other cues than olfactory cues.

Although NHPI can't be excluded, female BSF were most probably attracted to volatile compounds released from fermenting food. Compounds released during fruit fermentation, such as ethanol, acetic acid, ethyl acetate, and acetaldehyde (West, 1961), are known to attract fruit flies (Zhu *et al.*, 2003).

In addition, it also appears that, after a certain stage of degradation, food attractants become less attractive to females with fewer eggs laid. Indeed, the peak of attractiveness seems to overlap with the emission of VOCs during the first phases of decomposition. Thereafter, the biomass remaining to be degraded no longer attracts females, probably due to a change in the odour profile. This is consistent with the study of Rekinger (2020) who showed that at a certain stage of degradation, decaying organic matter contained in soil becomes less attractive to *Delia platura* females with fewer eggs laid.

Other studies have shown that cattle manure is a good egg-laying attractant for female BSF, as manure is the primary food for many insects in the wild, including black soldier fly larvae (Banks, 2014). The induction of oviposition activity by fresh cattle manure is related to the amount of odour produced and the moisture of the substrate, this made the fresh manure more attractive as the moisture helped produce more odour. The amount of odour impacts attraction for the female BSF to lay her eggs nearby, as it indicates a feeding opportunity for future larvae when the eggs hatch (Booth and Sheppard, 1984).

Our results showed that the rabbit feed used in this trial was the least attractive to BSF females. This is consistent with the studies of Banks (2014) and Diener (2010) who showed that using well moistened chicken feed proved to be relatively slow in attracting egg-laying BSF females probably because the fermentation process was slow and took a long time to generate the right type of odour.

Females are also strongly attracted by the eggs of their conspecifics (Zheng *et al.*, 2013), the volatiles of the bacteria species isolated from the eggs may be the cause of this attraction. In production situations it is clearly observed that females often choose to lay eggs in the same spot, crevice, where a first female laid its eggs, and the rest follows. The management of the attractant and technology to induce the females to lay their eggs on the desired egg matrices is a key design feature for industrial-level egg production.

VOCs from fruits such as banana (Zhu *et al.*, 2018), papaya (Kushwaha *et al.*, 2021), mango (Pino *et al.*, 2005), and pineapple (George *et al.*, 2023) have been extensively characterised. Many of these fruits share common VOCs such as α-Pinene, β-Pinene, Limonene, Myrcene, Linalool, and α-Terpinene. Among these compounds, α-Pinene, Limonene, and Linalool have been shown to attract egg-laying females of various fruit fly species (Antonatos *et al.*, 2021; Kamala Jayanthi *et al.*, 2012; Rasgado *et al.*, 2009). However, specific VOCs have also been identified for each fruit, such as soamyl acetate, phenylethanol, and 6-methyl-5-hepten-2-one in banana, benzyl isothiocyanate, (E,E)-α-marnesene, and methyl salicylate in papaya, and γ-terpinene, δ-3-carene, and ocimene in mango.

Some of these specific VOCs have been shown to attract egg-laying females of different insect species. For example, ethyl 2-methylbutanoate and γ-octalactone, produced by pineapple, have been demonstrated to attract egg-laying fruit flies (Becher *et al.*, 2010) and medflies (Tabilio *et al.*, 2013). Additionally, benzyl isothiocyanate, (E,E)-α-barnesene, and methyl salicylate emitted by papaya fruits have been shown to attract egg-laying females of cabbage flies (Buda and Radziute, 2008), apple maggots (Fein *et al.*, 1982), and fruit flies (Buda *et al.* 2009), respectively. Therefore, it is possible that these VOCs could play a role in attracting females of black soldier flies.

Several VOCs emitted from cattle manure are also known to attract egg-laying flies. For example, female stable flies (Jeanbourquin and Guerin, 2007), house flies (Shah *et al.*, 2016), are attracted to a variety of odours associated with cattle manure, including ammonia, carbon dioxide, and volatile fatty acids such as acetic acid, propionic acid, butanoic acid and butyric acid.

Fish waste also release VOCs that attract egg-laying flies. Some of the common VOCs produced by fish waste include trimethylamine, ammonia, acetic acid, and butyric acid (Wu and Bechtel, 2008). These VOCs are known to be attractant for various species of fruit flies (Colacci *et al.*, 2022; Delgado *et al.* 2022) and house flies (Geden, 2005).

The composition of rabbit feed comprises a variety of sources, such as hay, pellets, and grains. Consequently, the VOCs generated by rabbit feed are dependent on the specific ingredients used. A study investigating the VOCs produced by various animal feeds, including rabbit feed, revealed that they contain diverse compounds, such as aldehydes, ketones, and fatty acids (Yuan *et al.*, 2017). Grains commonly included in rabbit feed, such as corn, produce a range of VOCs, including aldehydes, ketones, and terpenes (Ukeh *et al.*, 2012). Nevertheless, research on the VOCs produced by rabbit feed and their attractiveness to flies is scarce. It is conceivable that some of the compounds generated by hay or grains may attract flies. However, the attractiveness of these compounds would depend on several factors, such as the specific compounds generated and their concentrations, as well as the presence of other competing odours in the environment.

Research has shown that black soldier fly females recognise a wide range of VOCs that are produced by fermenting organic matter, including acetic acid, butanoic acid, α-farnesene (Scieuzo *et al.*, 2021). However, the relative attractiveness of different VOCs can vary depending on the type and stage of the decaying organic matter. Additional research is required to characterise the diverse VOCs present within each substrate and to pinpoint the exact volatiles serving as chemoattractants.

Author contributions

Conceptualisation and methodology: Jamaa Zim and Rachid Bouharroud; software, formal analysis and data curation: Jamaa Zim, Halima Chkih and Patrick Lhomme; validation of protocols: Jamaa Zim, Mohammed Sarehane and Rachid Bouharroud; carrying out the experiment and collecting data: Jamaa Zim and Halima Chkih; writing-original draft preparation Halima Chkih, Jamaa Zim and Patrick Lhomme; writing-review and editing: Jamaa ZIM, Rachid Bouharroud and Patrick Lhomme; supervision and project administration: Mohammed Sarehane, Patrick Lhomme and Rachid Bouharroud.

Conflicts of interest

The authors declare no conflict of interest.

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