Exploring the Effects of the Volatile Chemical Indole on Black Soldier Fly Attraction and Oviposition Behavior

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ABSTRACT

Black soldier fly (BSF, *Hermetia illucens*) larvae are used worldwide for their ability to convert organic food scraps into valuable insect biomass and a digestate that can be used as a fertilizer and soil amendment. As such, BSF larvae are instrumental in the waste recycling industry. However, while there is considerable research dedicated to optimizing rearing of BSF larvae, very little is known about adult BSF behavior. Understanding adult behavior is valuable because it could improve adult mating and egg laying in rearing operations, ultimately leading to increased BSF production and more waste recycling. In this study, we tested if the addition of an attractive compound (indole) to an egg laying substrate would increase adult fly oviposition. We used a cage assay to measure BSF oviposition and landing rates when nearby indole. We documented the frequency that the BSF landed on wooden cutouts treated with indole and weighed the eggs left in those cutouts. Analysis of the results revealed that there was no statistically significant difference in BSF landing rates between indole-associated and control cutouts. However, BSF females laid significantly more eggs in indole-associated cutouts compared to control cutouts. These findings suggest that indole addition to the oviposition area does not increase the number of flies visiting but does increase the number of eggs laid by flies that do visit the site. As such, it is important to examine not just attraction, but also post-landing behavior, such as egg laying, which can also be influenced by chemical cues.

KEYWORDS: Black Soldier Fly, Indole, Oviposition, Attraction, Egg mass, Insect behavior

RICKY LE

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Kerry Mauck is an associate professor in the Department of Entomology. She received her Ph.D. from The Pennsylvania State University in 2012. In her current research, she works to discover and implement sustainable methods for controlling pests and pathogens in agriculture. A key part of this research is the study of black soldier fly (BSF) products as agricultural amendments that prime plant defenses against attackers.

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INTRODUCTION

Black soldier flies (BSF, *Hermetia illucens*) are used worldwide as biological recyclers because their larvae can ingest discarded, organic food scraps and transform them into useful outputs (Rehman et al., 2023). The BSF larvae do this by digesting food waste and converting it into two products: larval biomass (which can be used to feed livestock and pets) and a digestate that can be used as a fertilizer and soil amendment (Koyunoğlu, 2024; Fu et al., 2022). As such, BSF larvae are instrumental in the waste recycling industry. However, while there is considerable research dedicated to optimizing rearing of BSF larvae and their conversion of waste to usable products, we know very little about the behavior of adult BSF. Understanding adult behavior is valuable because it could improve adult mating and egg laying in rearing operations, ultimately leading to increased BSF production and more waste recycling. In this study, we tested if the addition of an attractive compound (indole) to an egg laying substrate would increase adult fly oviposition

Indole is a chemical often found in decaying matter and plants and has been proven to be attractive to other species of flies (Xing et. al., 2024). While there have not been any studies done to assess BSF attraction to indole, there have been several on other flies that act as decomposers, such as *Musca domestica* (common housefly). Recently, researchers discovered that this species has receptors for detecting indole, suggesting this compound may be important for finding food and oviposition sites (Pitts et. al., 2021). Previous research has also highlighted that these common house flies were able to recognize and were attracted to indole over other chemical attractants (Mulla et. al., 1977). Both houseflies and BSF rely on decaying organic matter as a food source. Therefore, it is reasonable to predict that BSF may also be able to recognize indole as a cue associated with resources. If indole is attractive, it could be used to help guide adult flies to preferred oviposition sites in commercial rearing operations. The more eggs are deposited on preferred sites, the more easily they can be harvested for use in waste recycling.

One way to assess BSF attraction to indole is to measure the effects of additions of this compound on BSF attraction to potential oviposition sites, and on their propensity to lay eggs on these sites. BSF will lay eggs on hard substrates, such as wood blocks or chips, near food waste, compost bins, and other sources of moist, organic materials that will later serve as a food source for BSF larvae. In our experiments to test if the addition of indole to oviposition sites, we used a standardized food diet, known as the Gainesville diet, as the food resource near the oviposition site. This food resource is composed of 50% wheat bran, 30% alfalfa, 20% corn meal, and water. To evaluate if indole is attractive and stimulatory of egg laying, we set up cages that mimicked natural BSF oviposition conditions. Each cage contained a tray with 200g of Gainesville diet and wood blocks affixed to the side for oviposition. Indole dispensers were added to the wood blocks in some cages, while others received a dispenser control with no indole. Flies (~300) were placed in the cages and observed for visitation behavior, then we evaluated eggs laid on the wood block.

METHOD

Our experiment utilizes a cage design that is based off of a previous study that examined BSF oviposition (Zheng et. al., 2013). To see if indole influenced oviposition rates, each cage was presented with one of three chemical treatments, which were placed inside vials attached onto the wooden cutouts. Vials contained a glass capillary tube so that odorants could flow into the cage and be detectable by BSF adults, but flies could not enter the vial to contact the indole. Every cage had two control (empty) vials as well as two of the treatment vials (40 mg indole, 80 mg indole, or a second set of empty vials) (Fig. 1). To reduce cage-specific bias, treatments were rotated between cages every day. For example, if a cage was being tested with 40 mg on Monday, then on Tuesday it will have 80 mg, and on Wednesday it will have untreated wooden blocks only. On Thursday, the cage would once again have 40 mg of indole, and the cycle would repeat. (Fig 2) In addition to the treatment, sugar-water solutions were provided in order to prolong fly lifespan. All flies were purchased as 5-day old larvae from EVO conversion Systems, LLC (College Station, TX, USA) and raised for two weeks on a Gainesville diet in a room with controlled temperature (27 ± 1°C) and humidity (70 ± 5%). Once they progressed to the pupa life stage , the pupae were collected and distributed into cages where they were allowed

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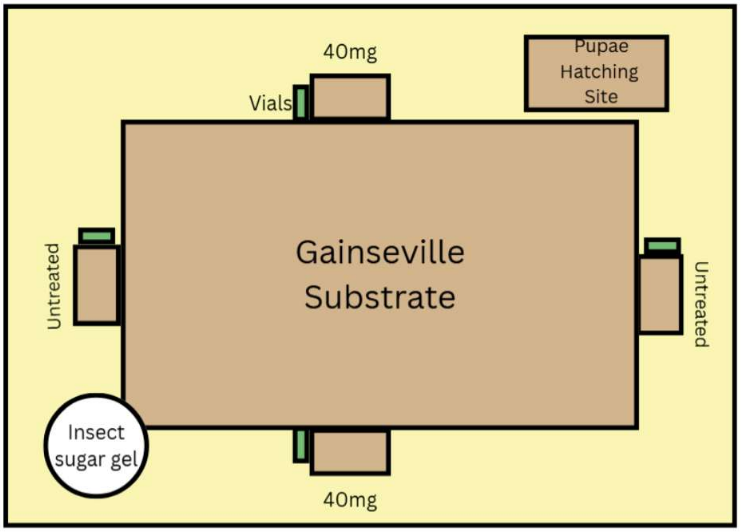

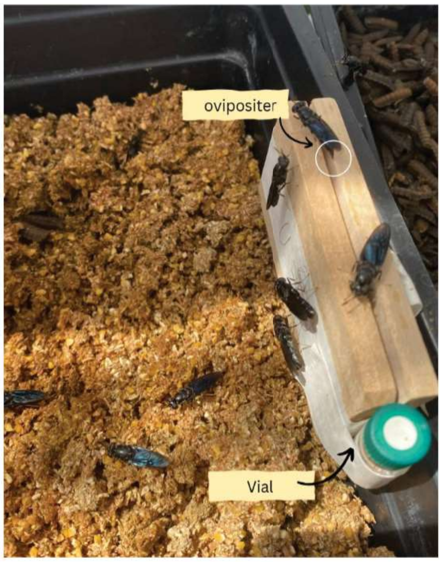

Figure 1. (A) An example of a choice test replicate. In this example, flies are given the choice to land on oviposition blocks (two pieces of poplar craft wood held together) with an indole dispenser attached to the blocks (in this case, 40 mg per vial) or oviposition blocks with empty dispensers. In the other set ups, the choices would be two 80 mg dispensers vs. two empty dispensers, and empty dispensers at all four oviposition block locations. Adults emerge from the tray containing pupae and tests were run when ~50 flies were present in each cage. The insect gel station provides a sugar solution as a gelatin substance, which flies ingest to obtain energy. - (B) BSF adults investigating the wooden cutout with the vials. The vial is taped onto the side. Here, we also see a female pointing her ovipositor toward the inside of the wooden cutout!

to hatch and interact with the cage. Once at least 50 flies were present 5 days, we began experimentation.

Our goals for these experiments were to determine: 1) if BSF adults are attracted to the chemical indole and 2) if the presence of indole near the oviposition site encourages more egg laying. To determine BSF adult attraction to indole, we observed adult behavior in the cages described above. Every time the BSF adult interacted with the wooden cutouts (which had the treatments in the vials attached), a tally was taken. BSF attraction to indole was determined based on how often the BSF adults interacted with the wooden cutouts and for how long (at least 1 minute or less than 1 minute). We set up 6 cages in total, with around 310 pre-pupae placed into every cage. Each cage was observed for 30 minutes every day, for 37 days, from April to May during the summer of 2024. This period was chosen for experimentation because BSF breed the most during warm months, although they can reproduce year-round (Ma et al., 2024). After around 5 days, the pre-pupae emerged as flies and once a critical mass of adult flies was reached, (50 flies), experiments commenced. Each cage was designated as one of the treatments: either 40 mg indole, 80mg indole, or no treatment (control). Each cage had 4 wooden cutouts in total, with two of them receiving vials with the treatment and two receiving only control (empty) vials (Fig. 1). After every observation period, we examined the wooden cutouts for eggs and removed eggs by gently scraping them into a container (Fig. 3). The eggs were weighed on an analytical balance. To ensure equal exposure, we rotated the treatments between cages each day so that every cage received each treatment at least once before any were repeated. For example, cage "A" might receive 40 mg on Monday, 80 mg on Tuesday, no treatment (control) on Wednesday, and then return to 40 mg on Thursday. This daily rotation ensured that all flies experienced every treatment in a balanced and consistent manner.

BSF emerge from their pupae shells at different rates, and so fly populations were different in each cage at any given moment. In addition, although each cage started with 310 pupae, not all pupae were able to successfully emerge. To help accommodate for this discrepancy between the cages, we converted our data from raw counts into a proportion instead, which measured the number of responding flies relative to the total number of flies within each cage.

We then conducted statistical analyses on these proportions instead of the raw numbers collected during each trial. This allowed comparisons among cages with slightly different numbers of flies.

We used the statistical analysis software Minitab in order to organize data into proportions and then used Microsoft Excel to conduct a two sample paired t-test and create graphs. The paired t-test analyzed the mean and standard deviation of the number of flies landing on either treated or control wooden cutouts, for each treatment. (40 mg, 80 mg, and untreated). A two-sample paired t-test was also used to analyze the egg data, with the mean egg mass left behind in the treated and untreated wooden cutouts being compared, for each treatment, respectively.

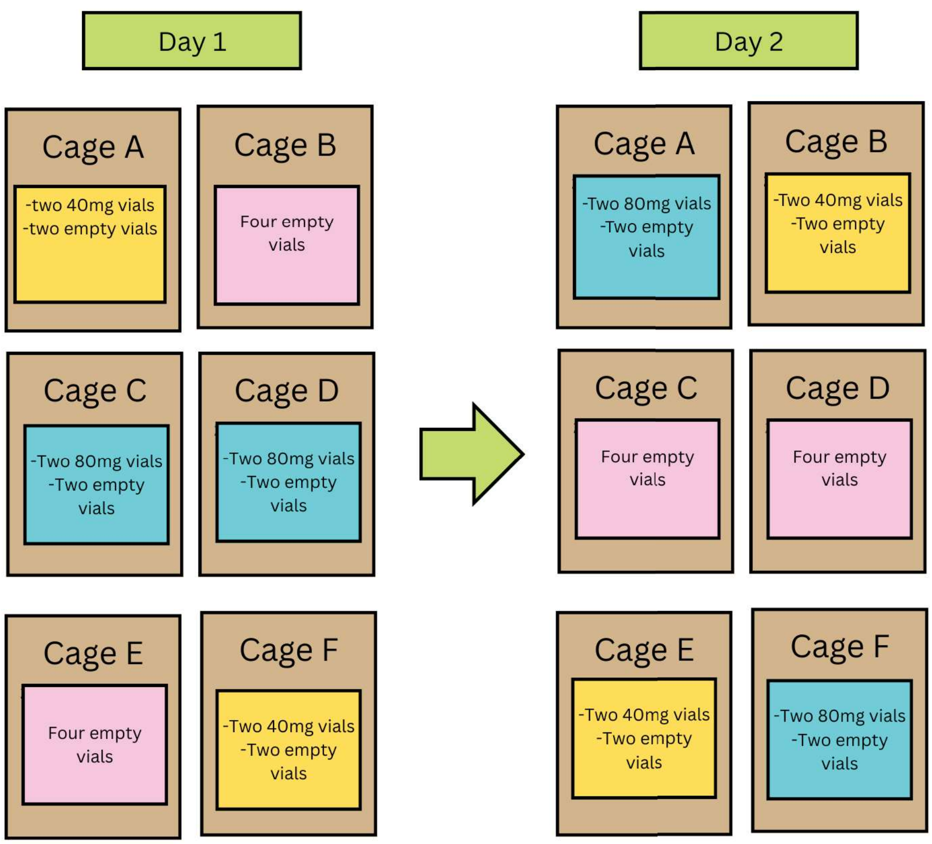

Figure 2. Treatments were rotated between cages every day to prevent any cage specific bias. Here, a sample rotation from day 1 and day 2 shown, where treatments are rotated between days.

Figure 3. (Left) BSF eggs deposited during an observation period. Yellow eggs can be seen on the insides of the wooden cutouts, where BSF females deposited eggs in the space between the pieces of wood. (Right) BSF eggs scooped up and placed onto a pre-weighed plastic container in order to measure egg weight.

RESULTS

BSF adult attraction to indole

We measured how often the adult flies landed on the wooden cutouts and how long they remained once a landing took place (Fig. 4). Looking at overall numbers of flies visiting indole- treated wood cutouts vs. untreated cutouts (controls), we found no difference in the percent of flies landing on each option (Fig. 4A and 4B). To examine if indole increases the number of flies spending more time on the cutouts (>1 minute), we considered only the flies that fell into this category. Again, we found no significant effect of either indole dose on the number of flies remaining on a cutout for more than a minute (Fig. 4C and 4D).

Effect of indole on oviposition

Egg mass results are shown in Fig. 5. We found that BSF preferred laying eggs in wooden cutouts treated with 40mg of indole, as opposed to the vials with 80 mg or the cage with all untreated vials. For cages with both the 80 mg or 40 mg of indole, the treated options had significantly more eggs compared to the untreated vials in those cages. The cage with 40 mg of indole had significantly more eggs laid on the 40 mg treated wooden cutout compared to untreated cutout and compared to the 80mg and untreated cages.

DISCUSSION

Analysis of the results revealed that there was not a statistically significant difference in the BSF landing on the 40 mg or 80 mg indole-filled vials compared to the empty control ones. However, BSF females laid significantly more eggs in wooden cutouts attached with vials filled with 40 mg of indole in comparison to 80 mg indole vials and untreated vials. These findings suggest that indole addition to the oviposition area does not increase the number of flies visiting, but does increase the number of eggs laid by flies that do visit the site. Our results indicate that it is important to examine not just attraction but also post-landing behavior, such as egg laying, which can also be influenced by chemical cues.

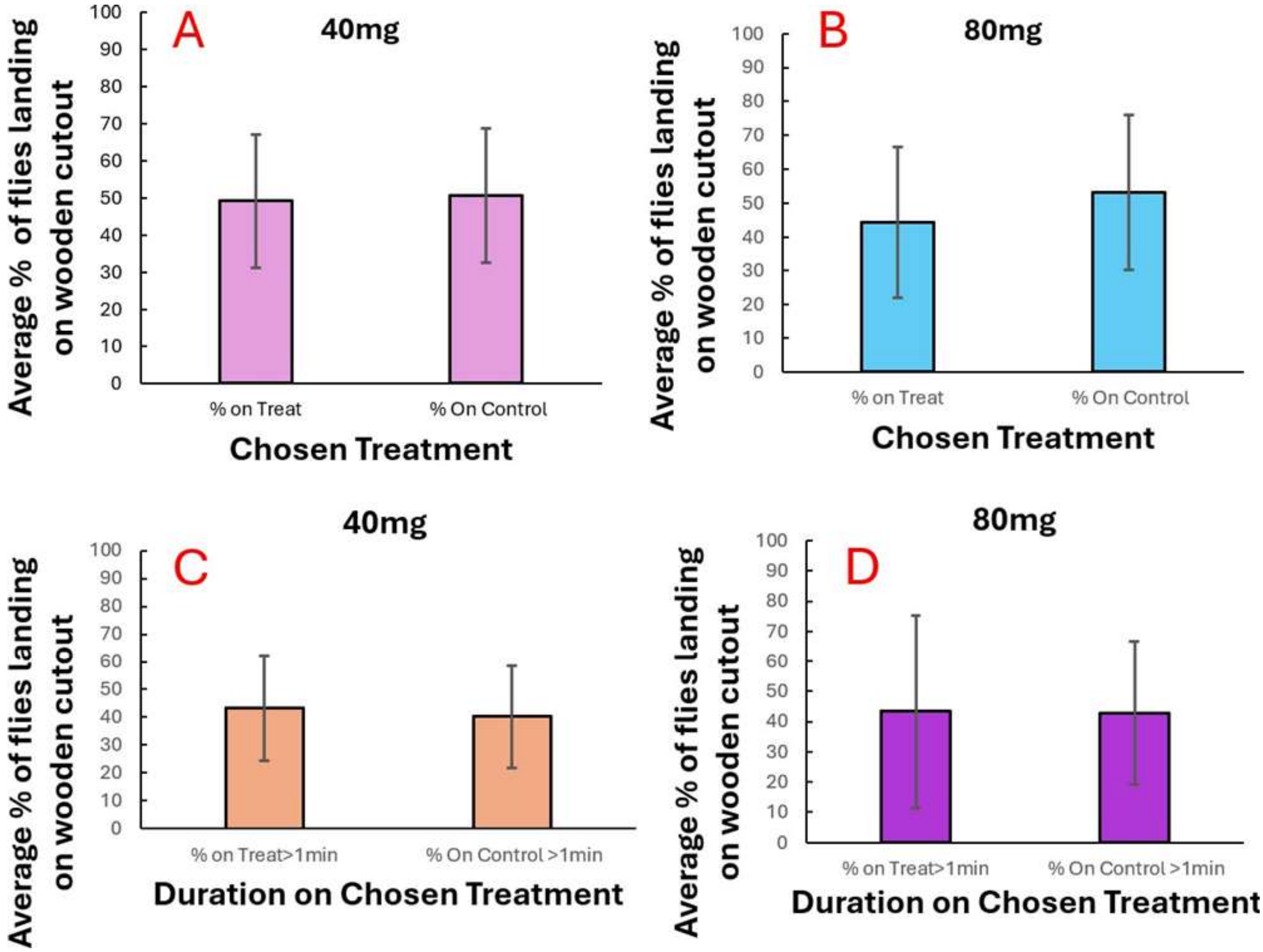

Figure 4. Bar charts depicting the % of flies landing on wooden cutouts per treatment. Bars are the mean percentage of flies across 31 trials, and error bars are the standard error.

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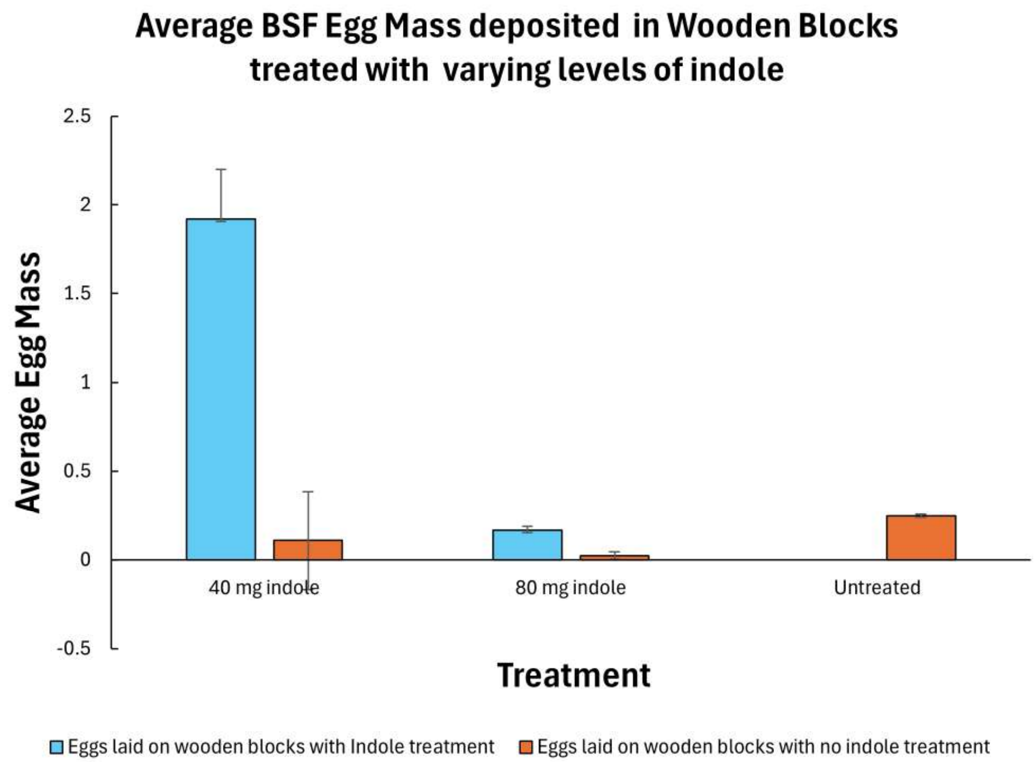

Figure 5. Average egg mass deposited on wood cutouts in different choice test set ups. The first set is 40 mg, with p-value of 0.05627. The second set is the eggs in the 80 mg cage, the p-value is 0.047358. For the eggs in the untreated cage, the p-value was 0.28097985.

We found that indole did not increase BSF adults landing on wooden cutouts emitting volatiles from vials 40 mg or 80 mg compared to untreated wooden cutouts. Additionally, these indole treatments did not increase the number of flies spending a longer amount of time on the wooden cutouts. As such, we can conclude that the adult flies are not differentially attracted to wooden cutouts with indole compared to those without them. However, we did find that, even though landing was not influenced, the likelihood of depositing eggs was. In the 40 mg cages, the p- value comparing egg mass left in treated vs. untreated wooden cutouts was 0.056274. While this is slightly above the standard $p < 0.05$ threshold, it suggests a propensity for oviposition with a low level of indole present. The 80 mg dose was also stimulatory for egg laying relative to the untreated cutouts in the same cage (p-value = 0.04736).

While only the 80 mg cage had statistically significant egg laying differences between the treated and untreated wood cutouts, there were significantly more eggs laid in the 40 mg cages overall, in both the treated and untreated wooden cutouts. This implies that the lower dose of indole may be closer to what the insects might experience with real decaying material, and therefore, may be more stimulatory for egg laying overall. The effect may also carry over to influence neighboring oviposition sites, since adjacent untreated wood cutouts also had more eggs than those in other cages with no indole or a very high dose of indole.

Our results indicate that treating oviposition sites with indole in industrial rearing operations may increase oviposition outputs. As shown in this study, using indole in controlled, small doses can promote egg-laying in BSF. More research is needed to determine the most effective dose, since our work also suggests that excessive amounts may have the opposite effect. This is seen in the difference in egg mass left behind in wooden cutouts treated with 40 mg of indole compared to those left in wooden cutouts treated with 80 mg. These results suggest that there may be a threshold effect, where higher concentrations become more repulsive than attractive to egg- carrying females.

Previous research has shown that stimulants like sugar, water, color, and even artificial light can be used to help increase BSF oviposition rates (Awal et al. 2022; Romano et al. 2020). This research expands on those findings by proposing the use of a chemical cue usually associated with larval food resources to increase egg oviposition rates. Specifically, we researched indole because it is a chemical found in several foods that BSF larvae can digest. These include cruciferous vegetables (Fujioka et al. 2016), pork (Pensabene & Fiddler, 1996), bread (Sayegh et al., 2023), and rice (Zeng et al., 2024). Indole is a very common stimulant that is found in several types of foods and has been proven to attract many species of flies (Mulla et al.1997; Pitts et al., 2021). Thus, scientists who look to optimize BSF rearing should look into doing more research with BSF and indole to better understand their relationship and the potential uses of indole in rearing productions.

While this research demonstrates that indole has an effect on oviposition rates, but further experiments are needed to determine if indole can be used to manipulate BSF oviposition behavior in rearing operations. In this experiment, the BSF adults did not show a preference for landing on wooden cutouts treated with 40 or 80 mg of indole compared to untreated controls. However, external factors such as sunlight exposure, indole diffusion rate, or even the presence of other flies may be affecting this decision. Future research efforts should build on our study by explicitly isolating these potentially important factors. For example, using devices that limit exposure to a subset of cues, such as a Y-tube olfactometer assay, will help researchers to understand which stimuli are most important for adult BSF oviposition decisions.

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