



# Stable Flies, *Stomoxys calcitrans* L. (Diptera: Muscidae), Improve Offspring Fitness by Avoiding Oviposition Substrates With Competitors or Parasites

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Oviposition site selection by gravid female insects is an important determinant in species distribution, abundance, and population dynamics. Females may assess the suitability of a potential oviposition substrate by using cues from conspecific or heterospecific individuals already present. Here, we assessed whether the presence of conspecific or heterospecific larvae and parasites influenced oviposition decisions by the stable fly, *Stomoxys calcitrans* (Linnaeus). Using dual and multiple-choice oviposition bioassays, we found that gravid female *S. calcitrans* avoided substrates with conspecific larvae, the larvae of house flies, *Musca domestica* (Linnaeus), and the mite *Macrocheles muscaedomesticae* (Scopoli). Avoidance of conspecific and heterospecific larvae persisted in the dark, suggesting that this behavior is mediated by chemical rather than visual cues. When we reared *S. calcitrans* in the presence of conspecific larvae and the larvae of house flies at different densities we found that this negatively affected emergence time, larval weight, larval survival, pupal weight, pupal survival, and adult weight. We also demonstrated that individuals of *S. calcitrans* developed in the presence of mites exhibited low egg hatchability, and poor larval and adult survival. Our study provides additional support for the “preference-performance” hypothesis in *S. calcitrans*, with gravid females preferring to lay eggs on a substrate that will enhance offspring fitness. We recommend that the chemical cues involved in avoidance by gravid female *S. calcitrans* of substrates with conspecific and heterospecific larvae should be elucidated. This could lead to the discovery of repellent chemicals important for *S. calcitrans* management.

**Keywords:** *Stomoxys calcitrans*, oviposition, competitors, parasites, offspring fitness

## INTRODUCTION

The fitness of holometabolic insects following metamorphosis is strongly influenced by the quality of the substrate in which they developed. Since the egg stage is immobile, gravid females need to lay eggs on a substrate that guarantees the fitness of their progeny. To select an appropriate breeding site, gravid females are guided by visual, mechanical, and chemical cues (Bentley and Day, 1989). In

addition to these, intra- and inter-specific competition (Michaud and Jyoti, 2007) and parasitism (Sadek et al., 2010; Kacsoh et al., 2013) are also known to influence oviposition decisions by gravid female insects. This influence can be either facilitatory, neutral or inhibitory (Prokopy et al., 2001). Facilitation of oviposition occurs when the presence of conspecific or heterospecific individuals in a specific substrate is an indicator of high quality habitat (Köhncke, 2013). For example, Gonzalez et al. (2016) demonstrated that female *Aedes aegyptii* and *Aedes albopictus* (Linnaeus) preferred to oviposit into water containing conspecific larvae. A neutral effect is evident when there is no observable cost of sharing the same habitat with other individuals. This has been shown in *Pieris napi* (Linnaeus), where females laid an approximately equal number of eggs when exposed to substrates with and without conspecific cues (Raitanen et al., 2014).

Inhibition of oviposition results when females are deterred from laying eggs on a substrate by the presence of competitors. Gravid females may avoid competition for their offspring through direct, aggressive interactions with other females, the marking and detection of larval resources with oviposition pheromones, or avoidance of cues associated with eggs or larvae already present. For example, females of the olive fruit fly, *Bactrocera oleae* (Rossi), display aggressive behavior toward each other (wing waving, fast running toward the opponent, pouncing and boxing on the head and thorax of the foe) when they are close to oviposition sites (Benelli, 2014). In the apple maggot, *Rhagoletis pomonella* (Walsh), females avoid larval competition by marking their oviposition site with a pheromone that deters most females from laying eggs (Averill and Prokopy, 1987). In the convergent lady beetle, *Hippodamia convergens* (Guérin-Méneville), gravid females lay more eggs on cleaned sorghum plants compared to those bearing conspecific and heterospecific larvae of the wheat aphid, *Schizaphis graminum* (Rondani) (Michaud and Jyoti, 2007). The inhibitory effect is mainly explained by the avoidance by insect gravid females of larval competition, which can reduce offspring growth, negatively affect survival (Yoshioka et al., 2012), and sometimes increase predatory cannibalism (Vijendravarma et al., 2013; Ahmad et al., 2015) due to the over-exploitation of resources. Similarly, inhibition of insect oviposition behavior can also be triggered by the presence of parasites. By laying more eggs on a substrate devoid of parasites, gravid female insects protect their offspring. This is evident in *Drosophila melanogaster* (Meigen), where females prefer to oviposit on alcohol-laden food sources that protect hatched larvae from wasp parasitisation (Kacsoh et al., 2013).

Stable flies, *Stomoxys calcitrans* (Linnaeus), are cosmopolitan blood-feeders that mechanically transmit viruses (e.g., West Nile fever virus, Rift Valley fever virus), bacteria (e.g., *Bacillus anthracis*, *Pasteurella multocida*), protozoans (e.g., *Trypanosoma evansi*, *Besnoitia besnoiti*), and helminths (e.g., *Habronema microstoma*, *Dirofilaria repens*) to their hosts. Hosts include cattle, camels, horses, dogs, and humans (Baldacchino et al., 2013). During periods of high abundance, *S. calcitrans* can reduce weight gain in cattle by up to 19%, and lead to a 40–60%

reduction in milk yields (Walker, 1990; Carn, 1996). In the USA, Taylor et al. (2012) estimated that *S. calcitrans* lead to economic losses of around \$2.2 billion per year.

Gravid females *S. calcitrans* use vertebrate herbivore dung for oviposition (Baleba et al., 2019), as well as rotting plant material, such as silage, hay, grass clippings, and garden compost (Cook et al., 2018). Decaying plant-based material is also an important ecological niche for other invertebrate species (Sladeczek et al., 2017) such as the house fly, *Musca domestica* (Linnaeus) (Broce and Haas, 1999) and the mite *Macrocheles muscaedomesticae* (Scopoli) (Jalil and Rodriguez, 1970). Similar to *S. calcitrans*, gravid females of *M. domestica* seek manure, decaying vegetation and compost for egg deposition (Machtinger et al., 2014). *Macrocheles muscaedomesticae* is a free-living cosmopolitan mite inhabiting decaying organic matter, including manure (Gerson et al., 2003). This mite feeds on the eggs and early larval stages of flies (Axtell, 1963). When reared on sterilized cow manure and fed with *M. domestica* eggs, Farahi et al. (2018) demonstrated that the longevity of *M. muscaedomesticae* can vary from 38.63 to 37.48 days under bisexual and oedipal reproduction, respectively. These mites are also found attached to the abdomen of adult flies, which they infect and parasitise during adult fly emergence from the puparium. This interaction has long been considered phoretic, but it is suspected that mites can extract haemolymph from their hosts, thereby reducing their flight performance (Luong et al., 2015). *Macrocheles* mites harm their hosts in different ways. Polak and Markow (1995) showed that these mites influenced the copulatory success in the fruit fly *Drosophila nigrospiracula* (Patterson and Wheeler). They demonstrated that males infested with two mites copulated less frequently than uninfested individuals. But after removing mites, previously infested males copulated as many times as flies with no history of infestation. Also, under laboratory condition, Polak and Polak (1996) showed that *Macrocheles* mites negatively affected the survivorship of *D. nigrospiracula*. The authors also demonstrated that this mite infestation lengthened the pre-oviposition period and reduced the number of eggs laid by gravid female *D. nigrospiracula*.

In this study, we determined how competition and parasitism affected oviposition decisions by gravid female *S. calcitrans*. We hypothesized they would avoid substrates with conspecific or heterospecific larvae and parasites to protect their offspring from intra- or inter-specific competition and parasitism. To test this hypothesis, we first assessed whether the presence of *S. calcitrans* larvae, *M. domestica* larvae and *M. muscaedomesticae* adults in a breeding substrate could influence oviposition decisions by *S. calcitrans*. Next, we addressed the ultimate evolutionary question of the fitness costs incurred by *S. calcitrans* immature stages when developing on a substrate with high competition or parasitism. These results provide insights into how gravid females of *S. calcitrans* are likely to behave in the presence of conspecific and heterospecific larvae, or parasites on a substrate. Such information is important for the management of *S. calcitrans* because it could lead to the discovery of semiochemicals acting either as attractants or repellents.

## MATERIALS AND METHODS

### Study Animals and Substrates

*Stomoxys calcitrans* flies were obtained from a single culture established for approximately 1 year at the Duduville campus of the International Centre of Insect Physiology and Ecology (*icipe*) in Nairobi, Kenya (1° 13' 12" S, 36° 52' 48" E; ≈ 1,600 m above sea level). To establish this colony, we used wild individual of *S. calcitrans* captured on the *icipe* campus using Vavoua traps. Prior to the experiment, gravid females of *S. calcitrans* from the established colony were exposed to rabbit dung (fermented in a plastic bag for 1 week) placed in plastic containers (21.5 × 14.5 × 7.4 cm) for oviposition. Rabbit dung was collected from the *icipe* rabbit breeding unit. After exposure for 24 h we transferred these containers to another cage (75 × 60 × 45 cm), and we monitored the development of the larval and pupal stages until adult emergence. Emerged flies were fed twice per day (800 and 1600 h) on defibrinated bovine blood obtained from a local abattoir poured on moistened cotton. Larvae of *M. domestica* were from a culture established from wild individuals captured from Kapiti Plain in Machakos County, Kenya (1° 37' 60" S, 37° 0' 0" E), using Vavoua traps (Mihok et al., 1995). They were reared on rabbit dung as previously described, but adults were fed on rotten banana, orange and watermelon. *Macrocheles muscaedomesticae* (Figure 5Ai) were harvested directly from unsterilized rabbit dung collected at the *icipe* rabbit breeding unit. We identified this mite following the morphological characteristics described by Özbek et al. (2015) and Kamaruzaman et al. (2018). Insect rearing and experiments were done in a laboratory under buffered conditions of 25 ± 5°C and 65 ± 5% relative humidity. The photoperiod was 12L:12D unless otherwise stated.

### Oviposition in the Presence of Conspecific and Heterospecific Larvae

We performed multiple-choice oviposition bioassays in cages (75 × 60 × 45 cm). In each cage, we allowed 20 gravid female *S. calcitrans* to oviposit on four types of substrate presented simultaneously: (1) rabbit dung, (2) rabbit dung with 10 *S. calcitrans* third instar larvae, (3) rabbit dung with 10 *M. domestica* third instar larvae, and (4) rabbit dung with 10 *S. calcitrans* and 10 *M. domestica* third instar larvae. For each replicate, we counted the number of eggs laid on each treatment 24 h after setting-up the bioassay. During all experiments performed in the laboratory, we used 50 g of rabbit dung, introduced in transparent 200 ml plastic cups. The high walls of these cups prevented escape of larvae from the dung. These bioassays were replicated 10 times. Here and in the following bioassays, flies in each replicate were used only once.

### Oviposition in Response to Abundance of Conspecific and Heterospecific Larvae

In the previous bioassay, we found that gravid female *S. calcitrans* avoided laying eggs on a substrate containing conspecific larvae (Figure 1A). Here, we aimed to establish whether avoidance of conspecifics was dependent on the number of individuals present on a substrate. We performed a density-dependent response oviposition bioassay where we exposed 20 gravid

female *S. calcitrans* to five rabbit dung substrates (as described previously) containing an increasing number (0, 10, 20, 30, and 40) of conspecific third instar larvae. We found that female *S. calcitrans* avoided all the substrates with conspecific larvae (Figure 1Bi). Afterwards, we assessed whether visual or chemical cues mediated conspecific avoidance. To do so, we followed the protocol developed by Yang and Shiao (2012) in two blow flies, *Chrysomya megacephala* (Fabricius) and *Chrysomya rufifacies* (Macquart). This involved two-choice bioassays in darkness (0L:24D photocycle) performed in a different climate room to the one where the culture was held so that the diurnal rhythm of the culture was not affected. In a cage (34 × 34 × 34 cm) containing 10 gravid female *S. calcitrans*, we introduced two treatments: (1) rabbit dung and (2) rabbit dung+40 third instar *S. calcitrans* larvae. We found that gravid female *S. calcitrans* significantly avoided the substrate containing conspecific larvae (Figure 1Bii). We also assessed whether gravid female *S. calcitrans* could avoid a substrate already used by conspecific larvae. In a two-choice bioassay, we allowed gravid female *S. calcitrans* to choose between used (where 40 *S. calcitrans* eggs were reared until the pupae stage and removed) and unused substrates (1 week fermented rabbit dung). Each bioassay described above was performed for 24 h after which we counted the number of eggs laid on each substrate treatment. Each bioassay was replicated 10 times.

The same series of experiments was then performed using *M. domestica* larvae. In the multiple-choice oviposition bioassay, we found that gravid females of *S. calcitrans* laid a similar number of eggs on the control substrate and the substrate with larvae of *M. domestica* (Figure 1A). We therefore sought to elucidate whether this behavior could change if the number of *M. domestica* larvae on the substrate increased, and to assess the role of visual or chemical cues in observed oviposition patterns using the same protocol as described for *S. calcitrans*.

In the previous experiments it may be that lower oviposition on substrates used by con- and heterospecific larvae results from reduced nutrient content as the substrates aged. To test this, we conducted two additional oviposition bioassays. In the first bioassay, we provided 20 gravid female *S. calcitrans* with access to three substrate types: (1) fresh rabbit dung, (2) rabbit dung that *S. calcitrans* larvae had developed in, and (3) rabbit dung from the same batch as 2 that no *S. calcitrans* larvae had developed in. The second bioassay was similar to the first one except that we used larvae of *M. domestica* instead. In both bioassays, we used 50 g of rabbit dung where 10 larvae were reared up to the pupal stage. We counted the number of eggs on each substrate after 24 h and we replicated the procedure 10 times.

### Oviposition in the Presence of Parasites

To evaluate how oviposition by *S. calcitrans* is affected by the presence of parasites in dung, we performed two-choice bioassays. Ten gravid female *S. calcitrans* were introduced to a cage (34 × 34 × 34 cm) containing a rabbit dung control and rabbit dung supplemented with 50 adults of the mite *M. muscaedomesticae*. We counted the number of eggs laid on each substrate after 24 h and we replicated the experiment 10 times. We found that *S. calcitrans* gravid females laid fewer eggs on the substrate with mites (Figure 2A). To find out if this behavior was

driven by visual or olfactory cues, we repeated the two-choice bioassays in darkness as described previously. Ten replicates of these bioassays were performed.

## Cost of Developing on a Substrate With Conspecific and Heterospecific Larvae

Danchin and Wagner (1997) postulated that individuals developing in groups would pay a fitness cost due to increased competition for limited resources. Therefore, we hypothesized that *S. calcitrans* offspring developing with conspecific or heterospecific larvae at high density would exhibit poor development. To assess the effect of intraspecific competition on *S. calcitrans*, we reared first instar larvae in groups of 5, 15, and 25 in plastic cups filled with 50 g of rabbit dung. Daily, we monitored the development of these larvae until adult emergence by recording the following parameters: (1) emergence time (from first instar larvae to adult emergence), (2) larval weight (weighed at 4, 7, and 10 days after the beginning of the experiment), (3) pupation percentage (equivalent to larval mortality), (4) pupal weight, (5) emergence percentage (equivalent to pupal mortality), and (6) adult weight. We replicated each bioassay 10 times. For the weight parameter, we individually weighed all larvae, pupae and adults that emerged from each density treatment. We weighed *S. calcitrans* larvae and pupae using a Sartorius balance (0.0001 g precision) after gently cleaning them with wet paper towel. To record the adult weight, we used a cylindrical container (height: 4 cm; diameter: 3 cm) to avoid their escape. After taring the container, we introduced a single adult inside and recorded its weight.

To test the fitness consequences for *S. calcitrans* developing in the presence of interspecific competition, we combined 10 first instar larvae of *S. calcitrans* with an incremental number of first instar *M. domestica* larvae as follows: 10:0, 10:10, 10:20, 10:30, 10:40. Each combination was introduced to 50 g of rabbit dung and replicated 10 times. Here, we also monitored the development of *S. calcitrans* larvae until adult emergence by recording the same six fitness parameters listed previously. Owing to our inability to differentiate *S. calcitrans* larvae from *M. domestica* larvae on day 4 and 7, we weighed *S. calcitrans* larvae only on day 10. On day 10, *S. calcitrans* larvae were smaller, with translucent bodies, in comparison with *M. domestica* larvae, which were larger and pale white to yellow in color.

## Cost of Developing in a Substrate With Parasites

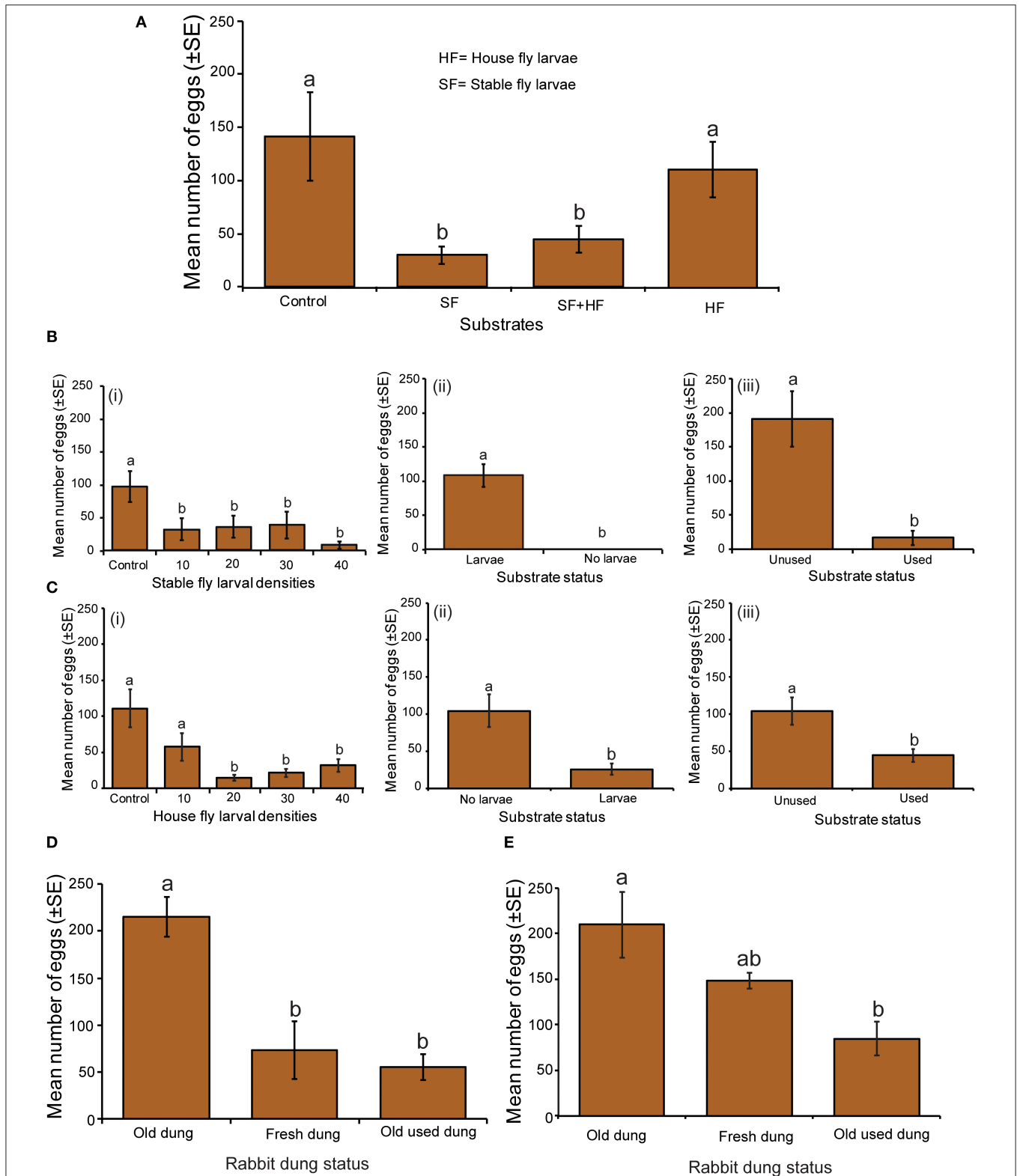
We hypothesized that if *S. calcitrans* eggs, larvae, or pupae were transferred to a substrate hosting the mite *M. muscaedomesticae*, egg hatchability, larval mortality, and adult survival in *S. calcitrans* would be affected. We introduced 10 eggs of *S. calcitrans* in transparent plastic cups of 200 ml prior containing 50 g of rabbit dung infested with 50 adult mites of *M. muscaedomesticae*. As a control, we transferred 10 eggs of *S. calcitrans* to rabbit dung without mites. Three days after, we determined the number of eggs that hatched by counting the number of larvae (L1 stage) found on each substrate treatment. To assess the effect of mite infestation on *S. calcitrans* larval

survival, we introduced 10 L1 larvae of *S. calcitrans* to rabbit dung hosting mites as previously described. For the control treatment, we also reared 10 L1 larvae on substrates without mites. In both treatments, we counted the number of dead larvae and the number of pupae. To determine adult survival, we transferred *S. calcitrans* pupae on rabbit dung infested with mites to initiate parasitism during adult emergence. After emergence, we collected 10 individuals of *S. calcitrans* loaded with at least five mites on the abdomen, and we transferred them to another cage (20 × 15 × 14.5 cm). As a control, we used 10 new emerged healthy flies (without mites). For 8 days, we supplied flies of each treatment (infested and non-infested) with defibrinated bovine blood daily and counted the number of dead individuals. We replicated the bioassay assessing egg hatchability and the larvae survival 10 times, whereas we replicated the bioassay testing adult survival five times.

## Data Analysis

All analyses were performed using the R environment for statistical computing (version 3.5.1) (R Core Team., 2018). The number of eggs laid in the presence of conspecific and heterospecific larvae, and with increasing abundance of *S. calcitrans* or *M. domestica* larvae, were not normally distributed (Shapiro-Wilk test:  $p < 0.05$ ). For this reason, we used Kruskal-Wallis tests to compare oviposition among presented substrates, followed by Dunn's *post-hoc* tests to identify homogenous subsets. Eggs laid under dark conditions, and with used or unused substrates were also not normally distributed. To compare oviposition under these conditions, we used the paired Mann-Wilcoxon test. In the bioassay with substrates of different age, the number of eggs laid were normally distributed and their variance were homogeneous (Bartlett's test:  $p > 0.05$ ), therefore we used the analysis of variance (ANOVA), followed by Student-Neuman-Keuls (SNK) *post-hoc* multiple comparison tests. The number of eggs laid by *S. calcitrans* in the presence or absence of the mite *M. muscaedomesticae* were also normally distributed and their variance was homogeneous, whether tested in the light or dark. To analyse these data, we used paired *t*-tests.

In our experiment assessing the effects of intraspecific competition on fitness parameters of *S. calcitrans*, emergence time and pupal weight data were not normally distributed (Shapiro-Wilk test:  $p < 0.05$ ). Due to this, we used Kruskal-Wallis tests followed by Dunn's *post-hoc* tests to see how emergence time and pupal weight varied across the tested larval densities. For the larval weight, except weight recorded at day 7, which was not normally distributed (we used the Kruskal-Wallis test followed by Dunn's *post-hoc* test for analysis), data from larval weight recorded on days 3 and 10 followed a normal distribution (Shapiro-Wilk test:  $p > 0.05$ ). Consequently, we ran analyses of variance (ANOVA) followed by Dunnett-Tukey-Kramer (DTK) *post-hoc* multiple comparisons tests (due to the inequality of the samples size) using the R library "DTK" (Lau, 2013). We also used ANOVA followed by DTK *post-hoc* tests to compare adult weight across the different larval densities owing to their normal distribution. Due to the binary nature of pupation (pupated vs. non-pupated) and adult emergence (emerged vs. non-emerged), we used generalized linear models (GLM) with



**FIGURE 1** | Influence of the presence of *S. calcitrans* and *M. domestica* larvae on oviposition decisions by *S. calcitrans*. **(A)** Bar chart showing the mean number of eggs laid by gravid female *S. calcitrans* in the presence of conspecific larvae and the larvae of the house fly (HF), *M. domestica*. **(B)** Bar charts illustrating the mean number of eggs laid by gravid female *S. calcitrans* in the presence of conspecific larvae following three experimental conditions: (i) different larval densities, (ii) in darkness, (iii) with unused and used substrates. **(C)** Bar charts depicting the mean number of eggs laid by gravid female *S. calcitrans* in the presence of

(Continued)

**FIGURE 1** | *M. domestica* larvae following three experimental conditions: (i) different larval densities, (ii) in darkness, and (iii) with unused and used substrates. Error bars indicate the standard error of the mean. Treatments with different lowercase letters are significantly different from each other (Kruskal-Wallis test followed by Dunn's *post-hoc* tests or Wilcoxon-Mann-Whitney tests,  $P < 0.05$ ,  $n = 10$ ). **(D)** Bart chart showing the mean number of eggs oviposited by gravid female *S. calcitrans* when presented with fresh rabbit dung, old rabbit dung and old rabbit dung used by *S. calcitrans* larvae. **(E)** Bart chart depicting the mean number of eggs oviposited by gravid female *S. calcitrans* when presented with fresh rabbit dung, old rabbit dung, and old rabbit dung used by *M. domestica* larvae.

binomial distribution to test whether these variables were affected by larval density (Warton and Hui, 2011). We established the significance of the model using analysis of deviance (with chi-squared test). Using the “emmeans” R package (Lenth, 2018), we ran Tukey's multiple comparisons tests to identify homogenous subsets in pupation and adult emergence among the larval densities.

In the bioassay testing the effect of interspecific competition on *S. calcitrans* fitness parameters, emergence time, and pupal weight data were not normally distributed (Shapiro-Wilk test:  $p < 0.05$ ), so we ran Kruskal-Wallis tests followed by Dunn's *post-hoc* tests to see which *M. domestica* larval densities differed from each other. Larval and pupal weight data were normally distributed (Shapiro-Wilk test:  $p > 0.05$ ), so we ran ANOVA tests followed by DTK *post-hoc* tests. We used a generalized linear model (GLM) with binomial distribution and the analysis of deviance test (with Chi-square test) followed by the Tukey's multiple comparisons tests to determine how pupation and the percentage emergence varied across the different larval densities.

For the bioassay aiming to assess the effect of mite infestation on egg hatchability and larval survival of *S. calcitrans* data were binary (hatched vs. unhatched). For this reason, we used a generalized linear model (GLM) with binomial distribution and an analysis of deviance (with chi-squared test). To compare survivorship of adult flies with mites and without mites, we performed Kaplan-Meier survival analysis with the Mantel-Cox log-rank chi-squared test using the R package “survival” (Therneau, 2015).

## RESULTS

### Oviposition in the Presence of Conspecific and Heterospecific Larvae

Female *S. calcitrans* deposited significantly more eggs on rabbit dung without conspecific larvae than on the substrates with conspecific larvae (**Figure 1A**;  $H = 11.45$ ,  $df = 3$ ,  $P = 0.009$ ). This avoidance behavior was not noted when the substrate was already colonized by the larvae of *M. domestica*. The number of eggs laid by female *S. calcitrans* on substrates with different conspecific larval density did not differ but was significantly lower than on uncolonized rabbit dung (**Figure 1Bi**,  $H = 14.13$ ,  $df = 4$ ,  $P = 0.007$ ). Under dark conditions, females completely avoided the substrate with conspecific larvae (**Figure 1Bii**:  $U = 0$ ,  $P < 0.001$ ). They also preferred the unused substrate over the substrate used by conspecific larvae (**Figure 1Biii**:  $U = 55$ ,  $P = 0.002$ ).

An incremental increase in the number of heterospecific larvae colonizing rabbit dung led to fewer eggs laid by female *S. calcitrans*. The number of eggs laid by female *S. calcitrans* was significantly lower on substrates with 20–40 *M. domestica*

larvae than on a control or dung with 10 heterospecific larvae (**Figure 1Ci**:  $H = 13.61$ ,  $df = 4$ ,  $P = 0.008$ ). Under dark conditions, female *S. calcitrans* laid significantly fewer eggs on a substrate with 40 *M. domestica* larvae in comparison with the control (**Figure 1Cii**:  $U = 55$ ,  $P = 0.002$ ). Similarly, significantly fewer eggs were laid on the substrate used for *M. domestica* development than the control (**Figure 1Ciii**:  $U = 42$ ,  $P = 0.019$ ).

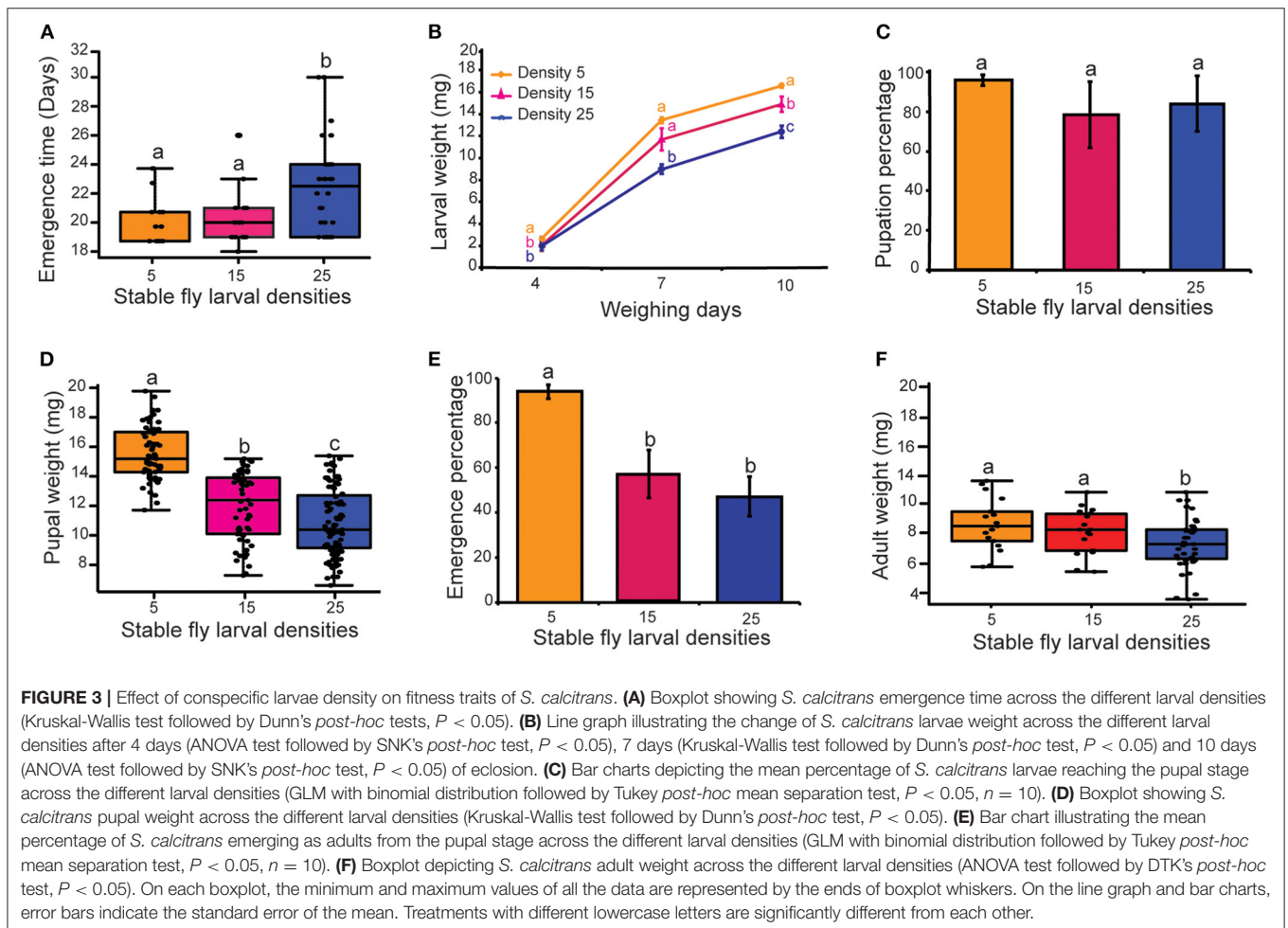
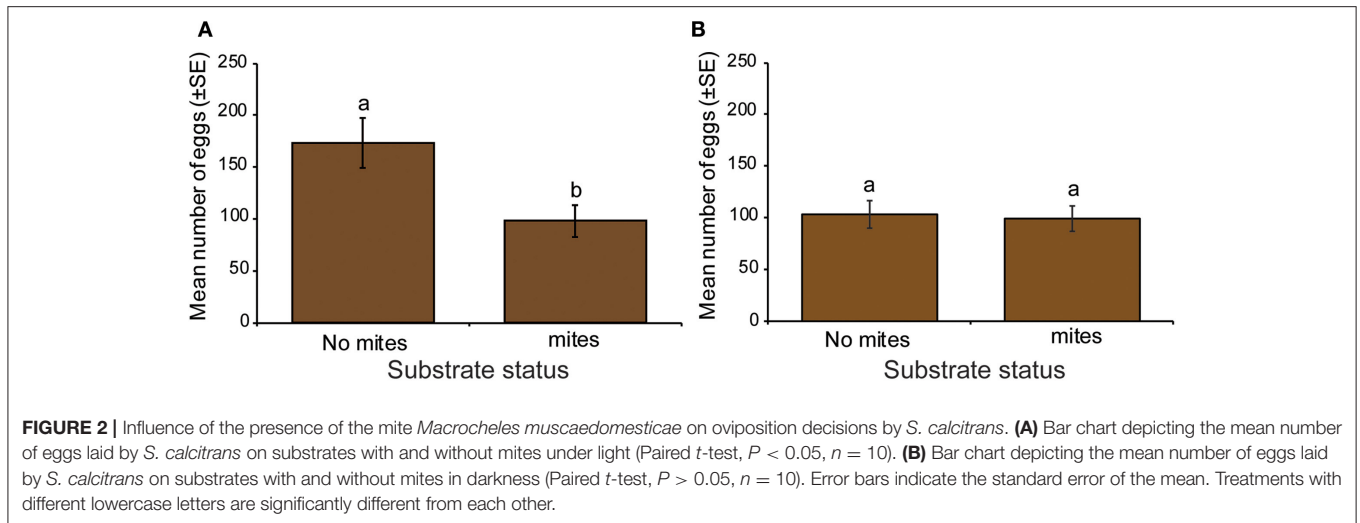
When investigating the role of dung age on avoidance of used substrates, the number of eggs oviposited by gravid female *S. calcitrans* varied significantly across the different rabbit dung treatments [**Figure 1D**:  $F_{(2,27)} = 14.62$ ,  $P < 0.0001$ , **Figure 1E**:  $F_{(2,27)} = 6.73$ ,  $P = 0.004$ ]. Female *S. calcitrans* laid significantly more eggs on old, unused rabbit dung than its counterpart used by *S. calcitrans* larvae. They also avoided fresh rabbit dung (**Figure 1D**). Likewise, these females laid fewer eggs on rabbit dung used by *M. domestica* larvae than on their unused counterpart (**Figure 1E**).

### Oviposition in the Presence of Parasites

Deposition of eggs by *S. calcitrans* on rabbit dung without or with mites differed. In the experiment performed under light, gravid female *S. calcitrans* laid significantly more eggs on the substrate devoid of mites than the substrate with mites (**Figure 2A**:  $t = -2.63$ ,  $df = 9$ ,  $P = 0.018$ ). Under dark conditions, the number of eggs laid by females did not differ between the two treatments (**Figure 2B**:  $t = -0.14$ ,  $df = 9$ ,  $P = 0.82$ ).

### Costs of Developing on a Substrate With Conspecific and Heterospecific Larvae Effect of Intraspecific Competition on *S. Calcitrans* Fitness Parameters

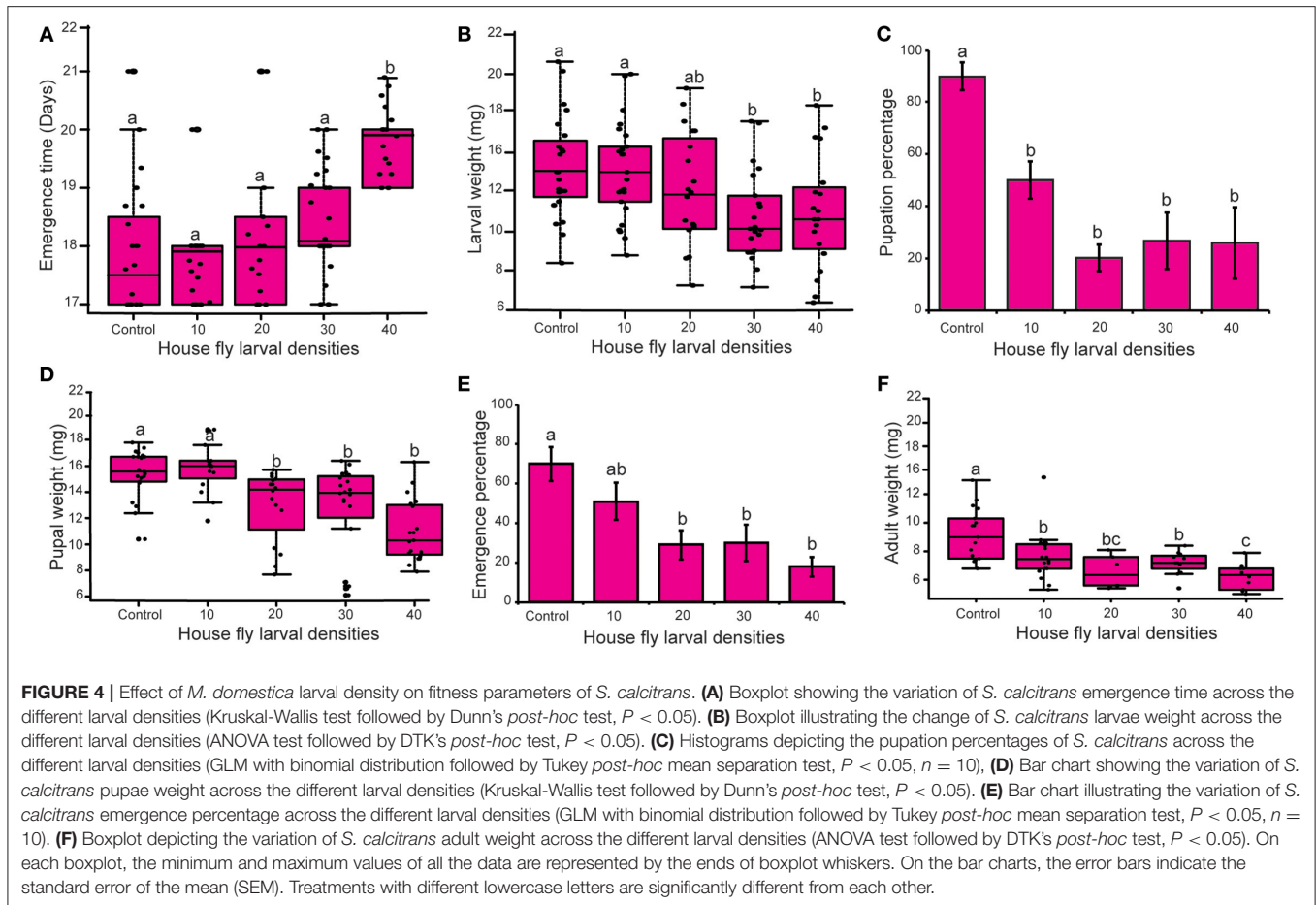
Larvae reared in a group of 5 and 15 reached the adult stage significantly faster than those reared in a group of 25 (**Figure 3A**;  $H = 7.73$ ,  $df = 2$ ,  $P = 0.02$ ). Also, larval density significantly influenced larval weight [Day 4:  $F_{(2-63)} = 3.5$ ,  $P = 0.035$ ; Day 7:  $H = 15.60$ ,  $df = 2$ ,  $P < 0.001$ ; Day 10:  $F_{(2-50)} = 20.91$ ,  $P < 0.001$ ]. Larvae reared in a group of 5, followed by those reared in a group of 15 were heavier in comparison with those reared in a group of 25 (**Figure 3B**). Despite differences in the body weight of larvae, the percentage of larvae reaching the pupal stage did not differ statistically (**Figure 3C**: GLM,  $\chi^2 = 2.27$ ,  $df = 2$ ,  $P = 0.32$ ). Pupal weight differed significantly across the larval density treatments (**Figure 3D**,  $H = 98.4$ ,  $df = 2$ ,  $P < 0.0001$ ), with pupal weight declining significantly with each incremental increase in larval density. The percentage of adults emerging from pupae reared in a group of 5 was significantly higher than at the higher larval densities (**Figure 3E**; GLM,  $\chi^2 = 17.06$ ,  $df$



$= 2$ ,  $P < 0.001$ ). Larval density also significantly affected adult weight [ $F_{(2-75)} = 4.50$ ,  $P < 0.05$ ]. Adults from larvae reared in a group of 5 and 15 weighed more than those reared in a group of 25 (Figure 3F).

### Effect of Interspecific Competition on *S. Calcitrans* Fitness Parameters

Density of *M. domestica* larvae significantly affected the emergence time ( $H = 13.88$ ,  $df = 4$ ,  $P = 0.007$ ), larval weight



[ $F_{(4-104)} = 4.45$ ,  $P = 0.002$ ], pupation percentage (GLM,  $\chi^2 = 44.51$ ,  $df = 4$ ,  $P < 0.001$ ), pupal weight ( $H = 35.54$ ,  $df = 4$ ,  $P < 0.001$ ), emergence percentage (GLM,  $\chi^2 = 24.38$ ,  $df = 4$ ,  $P < 0.001$ ) and adult weight [ $F_{(4-57)} = 7.95$ ,  $P < 0.001$ ] of the ten *S. calcitrans* individuals present in each substrate treatment. In comparison with *S. calcitrans* larvae reared in the presence of 0, 10, 20, and 30 *M. domestica* larvae, *S. calcitrans* larvae reared in the presence of 40 house fly larvae took longer to reach the adult stage (Figure 4A). When *S. calcitrans* were reared with 30 or 40 *M. domestica* larvae their larval weight was less than in other treatments (Figure 4B). The percentage of *S. calcitrans* larvae that reached the pupal stage was higher on the substrate devoid of house fly larvae (Figure 4C). Individuals from the substrate without larvae or with 10 *M. domestica* larvae had greater pupal weight (Figure 4D) and emergence percentage (Figure 4E) than those from other treatments. Adults from the substrate without house fly larvae were heavier than those reared from other treatments (Figure 4F).

### Cost of Developing in a Substrate With Parasites

The percentage of eggs that hatched was lower when placed on substrates hosting mites in comparison with substrates without mites (Figure 5B: GLM,  $\chi^2 = 59.55$ ,  $df = 1$ ,  $P < 0.001$ ). Survival

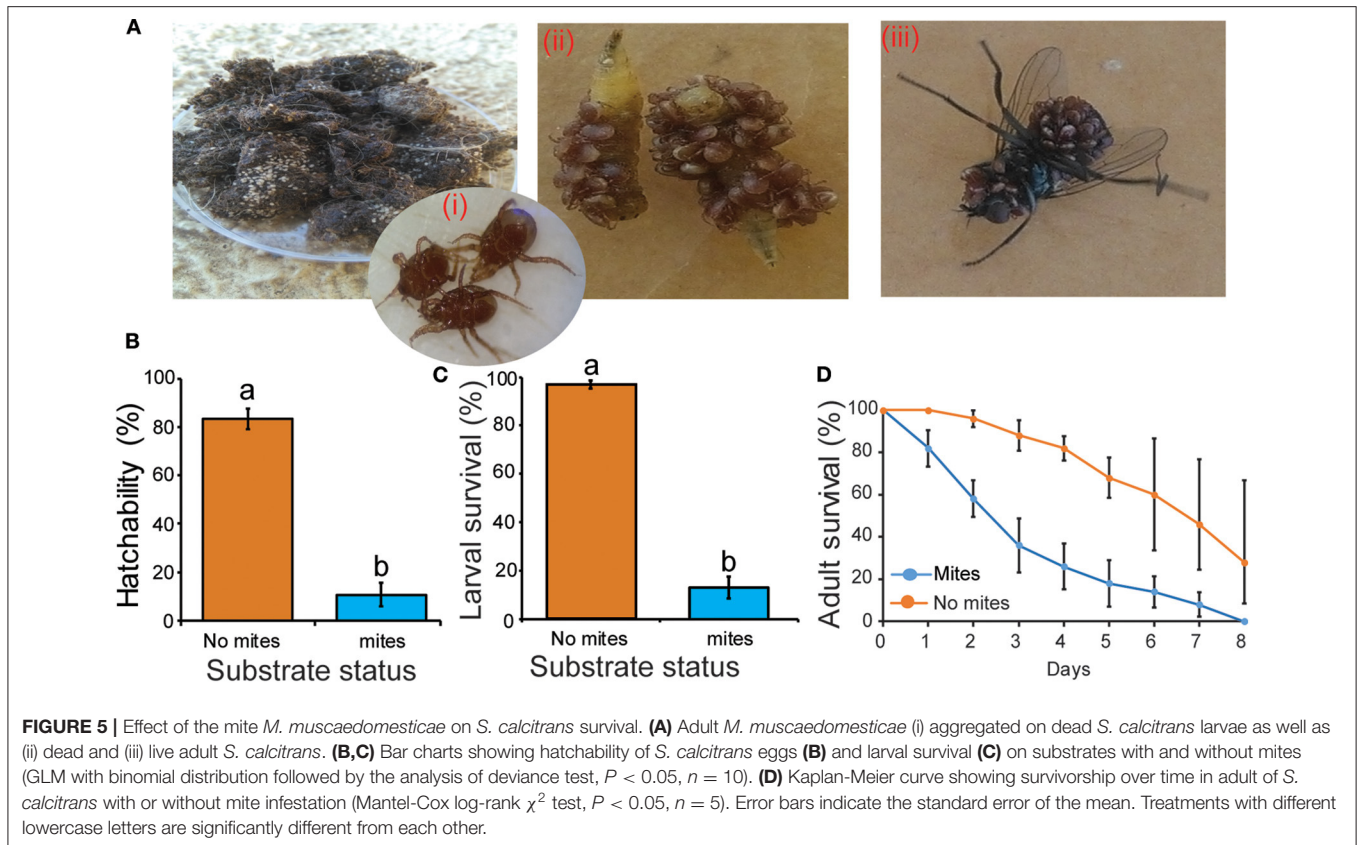
of larvae transferred to a substrate with mites was lower than those transferred to a substrate devoid of mites (Figure 5C: GLM,  $\chi^2 = 171.03$ ,  $df = 1$ ,  $P < 0.001$ ). Mite infestation of the substrate negatively affected adult survival (Figure 5D; log-rank test,  $\chi^2 = 6.4$ ,  $P = 0.011$ ).

## DISCUSSION

The results obtained in this work showed that oviposition decisions by gravid female *S. calcitrans* are influenced by the presence of conspecific, heterospecific larvae, and parasites on a substrate. Gravid female *S. calcitrans* laid significantly fewer eggs on substrates containing conspecific and heterospecific larvae. We demonstrated that 10 conspecific larvae were enough to trigger this avoidance, whereas in the presence of *M. domestica*, avoidance began when there were more than 10 larvae. This suggests that gravid female *S. calcitrans* consider not only the density but identity of competitors when selecting oviposition sites. Similarly, Wachira et al. (2010) demonstrated that substrate augmentation of *Culex quinquefasciatus* (Say) larval density significantly reduced the oviposition activity index of gravid female *Anopheles gambiae*.

In darkness, *S. calcitrans* laid significantly fewer eggs in substrates with conspecific and *M. domestica* larvae, suggesting





that instead of visual cues this avoidance is guided by chemical cues. Further, avoidance of oviposition substrates continued after larvae had been removed from them. The oviposition-deterrent effect of conspecific- and heterospecific-larvae found in this work has been shown in several species of Diptera. In *Culiseta longiareolata* (Macquart), 88% of gravid females laid eggs in pools with low conspecific larval density in comparison with pools with a high larval density (Kiflawi et al., 2003). In *Culex restuans* (Theobald), Reiskind and Wilson (2004) observed that gravid females avoided laying in containers with conspecific larvae. In *Chrysomya megacephala*, when liver with and without larvae of *Chrysomya rufifacies* were provided to gravid females, they oviposited on liver without larvae (Yang and Shiao, 2012). We suggest that *S. calcitrans* and *M. domestica* larvae may produce one or several chemical cues that strongly deter the oviposition behavior of gravid female *S. calcitrans*. Several chemical cues from conspecific- and heterospecific-larvae acting as repellents have already been identified in various studies. For instance, the alkane n-heneicosane found in the cuticle of *A. aegypti* larvae deters oviposition of this species at 100 and 1,000 mg/l (Seenivasagan et al., 2009). Gravid female *Aedes albopictus* are also repelled by n-heneicosane (secreted by *Ae. aegypti* larvae) at 30, 50, 100 and 200 ppm (Mendki et al., 2000). Several esters extracted from *A. aegypti* eggs (methyl dodecanoate, methyl tetradecanoate, methyl (Z)-9-hexadecenoate, methyl hexadecanoate (Z)-9-hexadecenoic acid,

methyl (Z)-9-octadecenoate, and methyl octadecanoate (Z)-9-octadecenoic acid) were found to repel *Ae. aegypti* gravid females (Seenivasagan et al., 2009). Frass produced by developing larvae may also represent a source of chemical cues used by *S. calcitrans* to avoid substrates. Frass of conspecific- and hetero-specific larvae has been found to repel a range of insects, including *Cydalima perspectalis* (Walker) (Molnár et al., 2017), *Monochamus alternatus* (Hope) (Li and Zhang, 2006), *Delia radicum* (Linnaeus) (Jones and Finch, 1987) and *Ostrinia furnacalis* (Guenée), *Ostrinia scapularis* (Walker), and *Ostrinia latipennis* (Warren) (Li and Ishikawa, 2004). Our observations offer the potential for discovery of repellent compounds that may be used to manage *S. calcitrans* and reduce the spread of diseases that they transmit.

We found that intra- or inter-specific competition negatively affected the fitness of *S. calcitrans* offspring. This provides a functional basis for avoidance of substrates with conspecific or heterospecific larvae by gravid female *S. calcitrans*. The impact of larval density on fitness parameters has also been found in other Diptera including *Hermetia illucens* (Barragan-Fonseca et al., 2018), *B. oleae* (Burrack et al., 2009), *D. melanogaster* (Durisko and Dukas, 2013), *Ae. albopictus* (Yoshioka et al., 2012) and *Ae. aegypti* (Reiskind and Lounibos, 2009). When resources are limited, larval crowding induces exploitative competition that lengthens larval developmental time, increases larval mortality, and reduces larval, pupal and adult weight (Sugiura et al., 2007; Legros et al., 2009). This effect is mainly attributed to a reduction

in the share of nutrients available in the breeding substrate for individuals. For illustration, Dutra et al. (2016) observed that glycogen concentration in *Ae. aegypti* larvae decreased by almost 50% as larval density increased. In *H. illucens*, crude fat concentration is higher in larvae reared at low density than those reared at high density (Barragan-Fonseca et al., 2018). Other studies suggest that high larval mortality observed with increased larval density results from interference competition. In crowded situations, *D. melanogaster* larvae exhibit cannibalism by killing and consuming other larvae (Vijendravarma et al., 2013; Ahmad et al., 2015).

Our study revealed that gravid female *S. calcitrans* were able to distinguish between substrates hosting parasites from substrates without them. In the light, gravid female *S. calcitrans* avoided the substrate containing the mite *M. muscaedomesticae*. Avoidance of parasitized substrates for oviposition is also found in other insect groups. Sadek et al. (2010) demonstrated that the moth *Spodoptera littoralis* (Biosduval) preferred to oviposit on alfafa compared to cotton where their eggs are more likely to be parasitized by the wasp, *Chelonus inanitus* (Linnaeus). In darkness, we observed that gravid female *S. calcitrans* deposited the same number of eggs on substrates with and without mites. This indicates that rather than chemical cues, *S. calcitrans* may use visual cues to detect mites on the substrate. In *D. melanogaster*, Kacsoh et al. (2013) showed that oviposition in an alcohol-laden food source that protects hatched larvae from wasps was mediated by visual cues. As in the case of substrate colonization by con- and heterospecific larvae, avoidance of *M. muscaedomesticae* also benefited the fitness of *S. calcitrans* offspring. We found that egg hatchability, and larval and adult survival were higher in substrates without the mite *M. muscaedomesticae*. Axtell (1986) reported that this mite could kill three to four *S. calcitrans* eggs per day. As well as hemolymph consumption when attached to larvae or adults, it is also suspected that on the host, the mite also induces melanisation which is an immune response involving the enzyme phenoloxidase. González-Santoyo and Córdoba-Aguilar (2012) indicated that the production of this enzyme has a fitness cost in the host. We suggest that these two factors contributed to higher mortality of *S. calcitrans* larvae and adults in the presence of mites. As such, *M. muscaedomesticae* may represent a viable biological control agent for *S. calcitrans*.

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In summary, our results indicate that gravid female *S. calcitrans* avoid substrates colonized by conspecific larvae, *M. domestica* larvae and the mite *M. muscaedomesticae*. This is likely due to the high fitness costs of competition and parasitisation for their offspring. We believe that the avoidance of substrates with conspecific and heterospecific larvae in *S. calcitrans* is mediated by chemical cues. Therefore, we recommend that additional studies be conducted to identify the chemical cues implicated in this avoidance behavior and their source. This will aid in development of improved control strategies against *S. calcitrans*.

## DATA AVAILABILITY STATEMENT

All data collected during this study are archived in Dryad data repository ([https://datadryad.org/stash/share/D1eo6OeGBa91y7SB\\_izKUH7WQ1ihnmsJxOQM5oy34Tg](https://datadryad.org/stash/share/D1eo6OeGBa91y7SB_izKUH7WQ1ihnmsJxOQM5oy34Tg)).

## AUTHOR CONTRIBUTIONS

SB conceived the research idea, designed the experiment, collected and analyzed the data, and wrote the manuscript. DM and BT supervised and proofread the manuscript. MG and CW designed the experiment, supervised, and assisted with manuscript preparation. All authors have read and approved the manuscript.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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