Olfactory preferences of gravid female stable flies, *Stomoxys calcitrans* L. (Diptera: Muscidae), and their fitness consequences

by

Bernard Steve Soh Baleba

Submitted in partial fulfilment of the requirements for the degree
Philosophiae Doctor in Entomology

in the

Department of Zoology and Entomology
Faculty of Natural and Agricultural Sciences

UNIVERSITY OF PRETORIA

July 2019

Olfactory preferences of gravid female stable flies, *Stomoxys calcitrans* L. (Diptera: Muscidae), and their fitness consequences

by

Bernard Steve Soh Baleba

Supervisors:

Prof. Christopher W. Weldon
Assistant Professor
Department of Zoology and Entomology
University of Pretoria
South Africa
Email: cwweldon@zoology.up.ac.za

Dr Merid Getahun
Scientist, Animal Health Theme
International Centre of Insect Physiology and Ecology (*icipe*)
Nairobi
Kenya
Email: mgetahun@icipe.org

Dr Daniel Masiga
Principal Scientist and Head
Animal Health Theme
International Centre of Insect Physiology and Ecology (*icipe*)
Email: dmasiga@icipe.org

Prof. Baldwyn Torto
Principal Scientist and Head
Behavioural and Chemical Ecology Unit
International Centre of Insect Physiology and Ecology (*icipe*)
Email: btorto@icipe.org

DECLARATION

I declare that the dissertation/thesis, which I hereby submit for the degree Doctor of Philosophy in Entomology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

ETHICS STATEMENT

The author, whose name appears on the title page of this dissertation/thesis, has obtained, for the research described in this work, the applicable research ethics approval.

The author declares that he has observed the ethical standards required in terms of the University of Pretoria's Code of Ethics for Researchers and the Policy guidelines for responsible research.

Bernard Steve Soh Baleba

July 2019

Thesis summary

Oviposition decisions are a prominent example of behaviour directly affecting the fitness, abundance, distribution, and population dynamics of holometabolous insects. Due to eggs being immobile and adult insects often not practicing biparental and/or maternal care, gravid females, when ovipositing, should select substrate(s) that maximise fitness of their offspring. Studies have revealed that this selection is influenced by biotic (intra and interspecific competition, parasitism, larval experience, etc…) and abiotic (olfactory cues, visuals cues, substrate physiochemical properties, etc…) factors. However, in the stable fly, *Stomoxys calcitrans* L. (Diptera: Muscidae), a cosmopolitan blood-feeder that transmits several pathogens to animals, the influence of these factors in gravid female oviposition decisions is still unknown. Therefore, in this thesis, I examined oviposition decisions by *S. calcitrans*, associated fitness consequences for offspring, the olfactory cue(s) involved, and the influence of biotic factors in egg-laying decisions.

I demonstrated (Chapter 2) that *S. calcitrans* prefer to lay on donkey and sheep dung over camel, cow, elephant, giraffe, and zebra dung or grass. I showed that this preference was related to the good nutritional value of these substrates that consequently led to the best performance of *S. calcitrans* offspring. Furthermore, I identified β-citronellene and (R)-(-)-carvone, from donkey and sheep dung, respectively, as the olfactory cues responsible for observed preference-performance behaviour. In the laboratory, β-citronellene and carvone elicited the strongest oviposition behaviour in *S. calcitrans*. I confirmed this in the field by showing that traps baited with these compounds also caught more gravid *S. calcitrans*.

To develop a better understanding of oviposition site selection in *S. calcitrans*, I went on to show (Chapter 3) that *S. calcitrans* avoided substrates with conspecific larvae, heterospecific (*Musca domestica*) larvae, or the mite, *Macrocheles muscaedomesticae*. This avoidance behaviour was associated with fitness costs to offspring incurred in the presence of competition and parasitism. Competition with conspecific larvae led to significant changes in wing morphology (Chapter 4), with flies reared in a group of 5 having larger wing centroid size, wing length, wing width, wing area and wing loading compared with those reared in a group of 25. Also, these parameters were higher in flies developed in donkey and sheep dung in comparison with those grown in camel and cow dung. Preferred dung may lead to adults with better dispersal capabilities. Larval experience does not affect oviposition decisions by female *S. calcitrans* because they did not prefer to oviposit on their developmental substrate.

This ruled out the Hopkins' host selection principle in *S. calcitrans* and verifies the importance of innate rather than learned oviposition preferences in this species.

Overall, the results reported in this thesis have enabled me to generate a fundamental knowledge of *S. calcitrans* oviposition decisions. Also, I have identified novel candidate attractants that could enhance trap catch of *S. calcitrans* to improve surveillance and limit transmission of pathogens. Furthermore, this thesis opens new research avenues in identifying repellents from conspecific and heterospecific larvae, which if packaged with the identified attractants could be used in a push-pull control system against *S. calcitrans*.

Dedication

To my daughter born exactly one week after the start of this work. Through this thesis, I hope you will understand and forgive my absence during the first three years of your life

Acknowledgements

I would like to thank my supervisors: Dr Merid Getahun, Prof. Christopher Weldon, Prof. Baldwyn Torto and Dr Daniel Masiga for all the support and freedom they have given me to think outside the box. Their sage advice, technical help, relentless enthusiasm and invaluable discussions positively contributed to the quality of this thesis. I am very grateful for that.

I am grateful to the German Academic Exchange Service (DAAD) that provided me with a PhD research fellowship through the African Regional Postgraduate Programme in Insect Science (ARPPIS) hosted at the International Centre of Insect Physiology and Ecology (*icipe*). My sincere appreciation also goes to the European Union that financially support the research activities of this thesis through the IBCARP camel, grant no. DCI-FOOD/2014/ 346-739 and Max Planck - icipe partner group for funding. I also acknowledge the University of Pretoria for permitting me to register in their PhD in Entomology programme.

Thanks to the Capacity Building and Institutional Development (CBID) staff most especially, Dr Robert Skilton, Vivian Atieno, Margaret Ochanda and Esther Ndungu for always being there to help with administrative matters.

To my ARRPIS classmates of 2016: Kieran Yisa, Caroline Nkung'u, Inusa Ajene, Bethelihem Mekonnen, Souleyman Diallo, Alfonse Mutibha, Celestin Ndayisaba, and Nancy Njeru, thank you for the strong friendship and the wonderful time we spent together in Nairobi.

Lastly, I would like to thank all my family and friends for their love and support over the last few years

Table of contents

CHAPTER 1 General introduction 1

1.1 Morphology 2

1.2 Life cycle 2

1.3 Economic importance 4

1.4 Control methods 5

1.4.1 Sanitation 5

1.4.2 Chemical control 6

1.4.3 Biological control 6

1.4.4 Sensory cue-based controls 7

1.5 Host finding 8

1.6 Oviposition site selection 9

1.7 Chemical ecology 11

1.7.1 Olfactory system: antenna, sensilla types and odorant receptors 11

1.7.2 Chemoreceptor function 13

1.8 Thesis rationale 17

1.9 Thesis aim and objectives 17

CHAPTER 2 Egg-laying decisions based on olfactory cues enhance offspring fitness in *Stomoxys calcitrans* L. (Diptera: Muscidae) 19

2.1 ABSTRACT 20

2.2 INTRODUCTION 21

2.3 MATERIAL AND METHODS 22

2.4 RESULTS 30

2.5 DISCUSSION 41

2.6 CONCLUSION 43

CHAPTER 3 Stable flies, Stomoxys calcitrans L. (Diptera: Muscidae), improve offspring fitness by avoiding oviposition substrates with competitors or parasites 44

3.1 ABSTRACT 45

3.2 INTRODUCTION 46
3.3 MATERIAL AND METHODS 48
3.4 RESULTS 53
3.5 DISCUSSION 61
3.6 CONCLUSION 64
CHAPTER 4 Effect of larval density and substrate quality on the wing geometry of Stomoxys calcitrans L. (Diptera: Muscidae) 65
4.1 ABSTRACT 66
4.2 INTRODUCTION 67
4.3 MATERIAL AND METHODS 70
4.4 RESULTS 74
4.5 DISCUSSION 86
4.6 CONCLUSION 87
CHAPTER 5 Larval experience of stable fly, *Stomoxys calcitrans* L. 1758 (Diptera: Muscidae) does not influence oviposition preference in gravid females 89
5.1 ABSTRACT 90
5.2 INTRODUCTION 91
5.3 MATERIAL AND METHODS 93
5.4 RESULTS 97
5.5 DISCUSSION 105
5.6 CONCLUSION 107
CHAPTER 6 General discussion 108
6.1 Implications 111
6.2 future directions 112
6.3 Closing remarks 113
CHAPTER 7 References 114
Appendix 145

RESEARCH OUTPUTS

Journal articles

Baleba SBS, Torto B, Masiga D, Weldon CW and Merid N. Getahun. (2019). Egg-laying decisions based on olfactory cues enhance offspring fitness in *Stomoxys calcitrans* L. (Diptera: Muscidae). Scientific Reports, 9:3850.

Baleba SBS, Torto B, Masiga D, Getahun MN and Weldon CW. Stable flies, *Stomoxys calcitrans* L. (Diptera: Muscidae), improve offspring fitness by avoiding oviposition substrates with competitors or parasites. **Submitted (*BMC Ecology* Journal).**

Baleba SBS, Masiga D, Torto B, Masiga D, Weldon CW, and Merid N. Getahun (2019) Effect of larval density and substrate quality on the wing geometry of *Stomoxys calcitrans*, Linneaus 1758 (Diptera: Muscidae). Parasites & Vectors. 12: 222.

Baleba SBS, Weldon CW, Masiga D, Torto B, and Getahun MN (2019) Larval experience of stable fly, *Stomoxys calcitrans* Linneaus, 1758 (Diptera: Muscidae) does not influence oviposition preference in gravid females. Ecological Entomology.

Oral presentations

Baleba SBS, Torto B, Masiga D, Weldon CW and Merid N. Getahun. (2018). Herbivore dung-derived odours as bait for the stable fly *Stomoxys calcitrans* L. (Diptera: Muscidae). 9th International Congress of Dipterology. Windhoek/Namibia. 25-30 November 2018.

Poster presentations

Baleba SBS, Masiga D, Torto B, Weldon CW and Merid N. Getahun. (2017). "Mother knows" best hypothesis: case study in stable fly *Stomoxys calcitrans* L. (Diptera: Muscidae). Governor council meeting, icipe, Nairobi/Kenya. 20 November 2017.

Baleba SBS, Torto B, Masiga D, Weldon CW and Merid N. Getahun. (2018). Dung-derived odours as bait for stable fly *Stomoxys calcitrans* L. (Diptera: Muscidae). "Vector ecology and Disease" workshop, icipe, Mbita/Kenya. 30-July-03 August 2018.

CHAPTER 1 General introduction

1.1 Morphology

Native to Africa, and commonly known as the stable fly, *Stomoxys calcitrans* Linnaeus, 1758 (Diptera: Muscidae), is a synanthropic (Dsouli-Aymes et al. 2011a), cosmopolitan blood-feeding insect. The genus *Stomoxys* Geoffroy, comprises 18 currently identified species (Zumpt 1973; Skidmore 1985; Carvalho 2005). The species *S. calcitrans* (Fig. 1.1A) is morphologically similar to the house fly, *Musca domestica* Linnaeus, 1758 (Diptera: Muscidae) (Fig. 1B). However, three characters differentiate it from the latter: (1) the mouthparts are modified into a long, thin and black proboscis that serves to pierce the skin and suck the blood of hosts, (2) four longitudinal black lines are present on the dorsal part of the thorax, and (3) seven black spots are present on the dorsal part of the abdomen (Roungthip Masmeatathip et al. 2006; Salem 2012). Females and males are very similar; they measure 4 to 7 mm in length. However, the eyes of females are smaller and more widely separated than in the males (Fig. 1.1C).

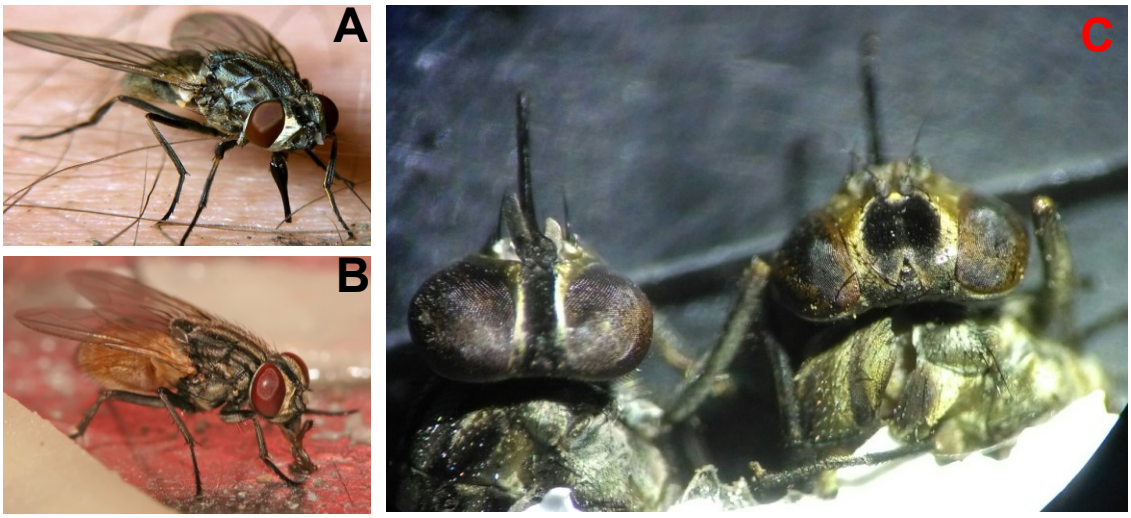

Figure 1.1. Morphology of adult *S. calcitrans*. (A) Adult of *S. calcitrans* (Photo: Bruce Marlin), (B) Adult of *M. domestica* (Photo: Muhammad M. Karim), (C) Female dioptic eyes (right) and male holoptic eyes (left) (Original photos).

1.2 Life cycle

Stomoxys calcitrans has a holometabolous life cycle, which passes through the egg, larval, pupal, and adult stages (Fig. 1.2). Newly emerged females require a blood meal (11-15 µL) for the maturation of their eggs (Schowalter et al. 1979). Blood-fed females begin to oviposit 4-8 days after copulation; they lay batches of 25-50 eggs during a period of around 20 days in decaying matter such as silage, crop residue, hay, grain, manure and soiled animal bedding

(Cançado et al. 2013; Salem 2012). Romero et al. (2006) found that the development of stable fly larvae depends on the bacterial composition of the oviposition medium. Females can select oviposition sites based on the microbial composition of decaying matter (Romero et al. 2006). Most eggs hatch after 24 hours. The emerged larvae are white and cylindrical; they go through three instars during 3 to 10 days before pupating. Puparia measure around 6 mm and progressively change their colour from white to dark. The adult emerges from the puparium after 6 to 8 days. The entire life cycle is influenced by temperature, relative humidity and photoperiod. Salem et al. (2012) established that the average cycle from egg to adult is 19.2±1.7 days and mean longevity of adults is 9.3±5.8 days when the colony is maintained at 25±2°C, 50 ± 10 % RH with a 12:12 photoperiod. Gilles et al. (2005) demonstrated that the survival of *S. calcitrans* immature stages is optimum between 20-25°C. *Stomoxys calcitrans* is known as a diurnal species with a peak in activity between 08:00 to 10:00 a.m. (Masmeatathip et al. 2006). Populations are multivoltine and their generation time over the year is principally dependent on temperature (Dsouli-Aymes et al. 2011). The abundance of *S. calcitrans* adults is highest during the rainy season (Dawit et al. 2012; Keawrayup et al. 2012).

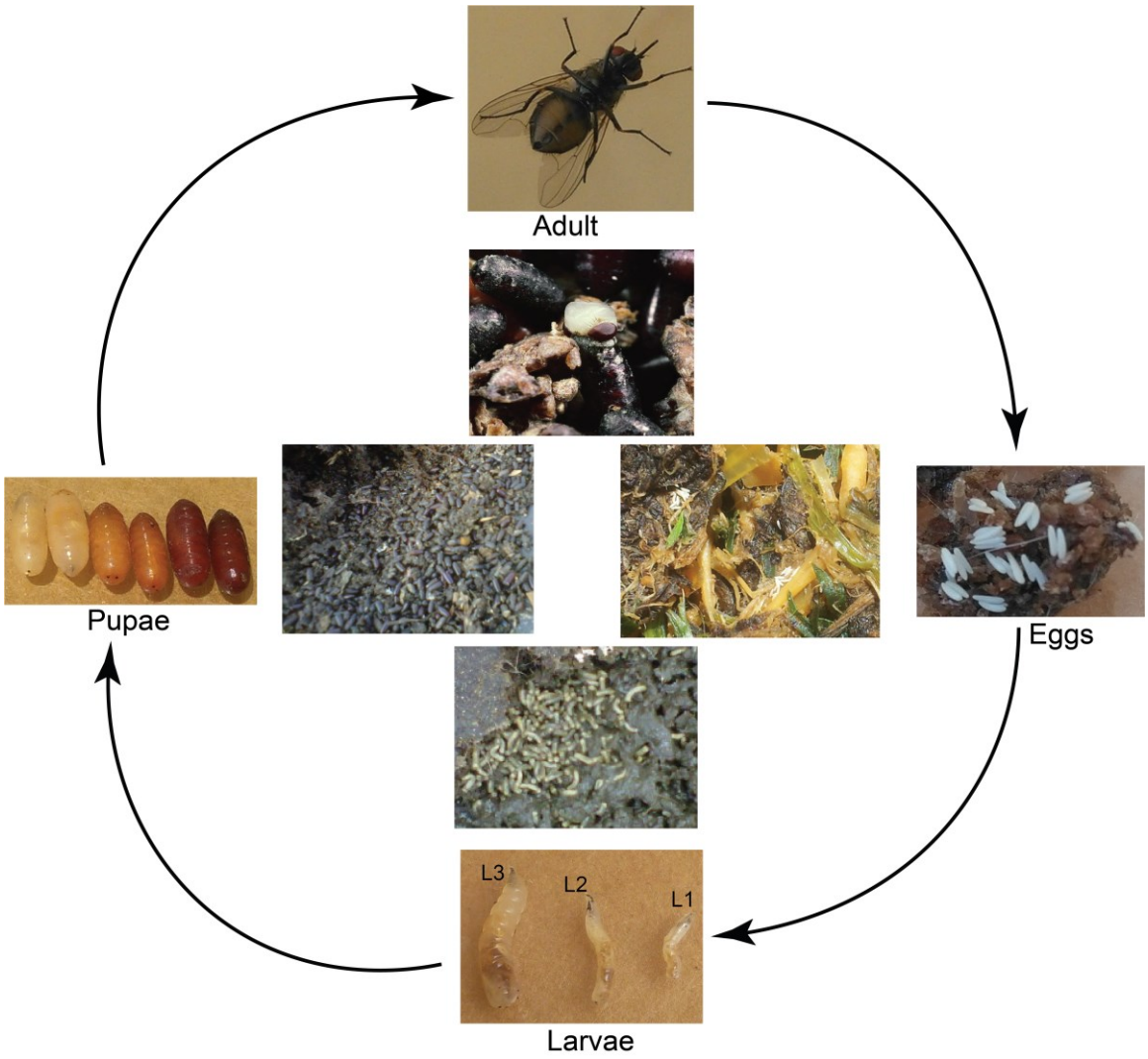

Figure 1.2. Life cycle of *S. calcitrans* (Original conception)

1.3 Economic importance

For the success of their mating, survival and egg production, female and male stable flies depend on repeated blood meals coming from several hosts, including cattle, horses and sheep (Bishopp, 1913; Broce et al., 2005), camels (Njiru et al. 2001; Tekle and Abebe 2001), and humans (Yeruham and Braverman, 1995). However, Higgins (1986) revealed that *S. calcitrans* prefer to feed on camel blood more than horse blood. *Stomoxys calcitrans* can consume on average of 11-15 µL of blood per meal (Schowalter et al. 1979). During their blood meals, which generally occur on the belly and the limbs of their hosts (Fig. 1.3), these flies can cause different injuries on their hosts. Baldacchino et al. (2013) classified the effects of *S. calcitrans* on their host into two groups: (1) direct effects, including annoyance, skin lesion, blood loss, stress, immunosuppression and reduction of food intake; and (2) indirect nuisance, including transmission of viruses [West Nile fever virus (Johnson et al. 2010), Rift

Valley fever virus (Turell et al., 2010)], bacteria [*Bacillus anthracis* (Hugh-Jones and Blackburn 2009), *Pasteurella multocida* (Krinsky 1976)], protozoa [*Trypanosoma evansi* (Sumba et al. 1998), *Besnoitia besnoit* (Sharif et al. 2019)], and helminths [*Habronema microstoma* (Traversa et al. 2008), *Dirofilaria repens* (Krinsky 1976)]. During outbreaks, *S. calcitrans* can reduce weight gain in cattle by up to 19%, and lead to a 40-60% reduction in milk yields (Carn 1996; Walker 1990). Surra disease, caused by the protozoan *T. evansi*, can cause morbidity of up to 30% and mortality of around 3.0% in camels (Njiru et al. 2001; Tekle and Abebe 2001). In the USA, Taylor et al. (2012) revealed that economic losses attributed to *S. calcitrans* infestation are estimated to be around $2.2 billion per year.

Figure 1.3. Infestation by *S. calcitrans* (Photo: David Cook)

1.4 Control methods

Stomoxys calcitrans control is currently focused on sanitation, chemical and biological control.

1.4.1 Sanitation

The procedure consists of reducing fly infestation by removing or dispersing any substrate that can serve as a developmental site (rotting straw and manure) so that it dries out before

maggots complete their development. This action provides a marginal control because all the *S. calcitrans* breeding sites are unlikely to be screened.

1.4.2 Chemical control

Several chemicals with different application methods have been evaluated for controlling *S. calcitrans* adults. Mount et al. (1966) found an 80% reduction in *S. calcitrans* populations when using Baygon (Bayer39007, methyl carbamate), naled, and fenthion at 1, 2, and 4% concentration, respectively. Hogsette and Ruff (1986) observed that flucythrinate and permethrin were effective against *S. calcitrans* for 10 weeks. Guglielmone et al. (2004) after having treated cattle with organophosphate found no significant decrease in *S. calcitrans* numbers. Insecticide resistance in stable flies has been detected among organochlorine and organophosphate insecticides (Somme 1985; Cilek and Greene 1994). Pitzer et al. (2010) identified several field populations of *S. calcitrans* in Florida that were resistant to permethrin, the most commonly used insecticide for stable fly management. As well the issue of resistance, pesticides have the disadvantages of being costly, toxic and non-ecologically selective.

1.4.3 Biological control

Biological control of *S. calcitrans* is mainly based on the utilisation of parasitoids (Fig. 1.4A) and entomopathogenic fungi (Fig. 1.4B). Works conducted by Jones and Weinzierl (1997) in the state of Illinois, USA, revealed that *Spalangia spp* can parasitize 93 % of *S. calcitrans*. Greene et al. (1989) found that during the winter on diaries in the northwerstern Florida, *Spalangia cameroni* parasitation is 76% in *S. calcitrans*. The study conducted by Skovgård and Jespersen (1999) in pig and cattle farms in Danmark showed that *Muscidifurax spp*. paratitize *S. calcitrans* at a rate of 11%. Using strain from Cornell University, Watson (1995) obtained 70% *S. calcitrans* mortality due to the entomopathogenic fungus *Beauveria bassiana*. In Spain, Moraes et al. (2008) found that larvae and pupae of *S. calcitrans* are not susceptible to *Metarhizium anisopliae*, but conidial suspensions concentrated at 10^{-7} and 10^{-8} cause egg mortality of 96.25% and 100%, respectively. Weeks et al. (2016), when testing a commercial formulation of different strains of *B. bassiana* and *M. anisoplae*, found a significant increase in the mortality of *S. calcitrans* from Florida university cultured colony.

The mite *Macrocheles muscaedomestica* (Fig. 1.4C) is also a potential biological control agent. This mite feeds on fly eggs and early larval stages (Axtell 1963). In adult flies, *M.*

muscaedomesticae individuals extract haemolymph from their hosts, which reduces fly survival.

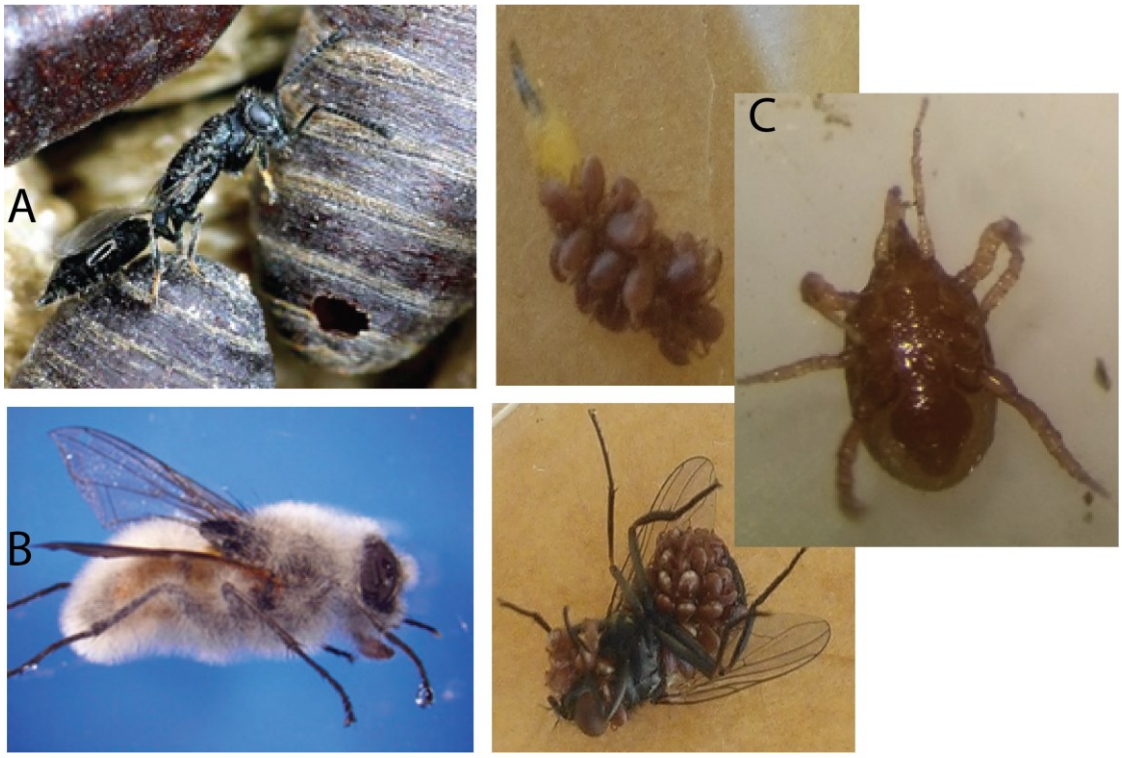

Figure 1.4. Biological control agents of *S. calcitrans*. (A) Emerged *Spalangia* spp (Photo: David Cook). (B) *Stomoxys calcitrans* individual killed by an entompathogenic fungus (Photo: David Cook). (C) *Macrocheles muscaedomesticae* infestation on larval and adult *S. calcitrans* (Original photo)

The implementation of the above control methods is widely influenced by many factors. Pitzer (2010) states that they rely on the season, temperature, type of substrate and moisture therein, host species composition, and the depth in substrates at which hosts (larvae and pupae) are found.

1.4.4 Sensory cue-based controls

Besides the classical control methods described above, behavioural control is another approach for managing insect pests that manipulates their responses to visual and olfactory stimuli to achieve population suppression. Insects exploit semiochemical compounds through their sense of smell to locate a suitable sexual partner and breeding host, to detect the presence of their predator, and to search for food (Norin 2007; Logan and Birkett 2007; Ali et al. 2015). The identification of the chemical cues involved in these interactions and

their use as lures constitute a promising tool in insect pest management (Khan et al. 2016; Logan and Birkett 2007; Norin, 2007). Mihok et al. (1995) demonstrated baiting Vavoua traps with 1-octen-3-ol increased *S. calcitrans* catches up to 3.7 times. Tangtrakulwanich et al. (2015) demonstrated that traps with a binary blend lure of phenol and m-cresol or p-cresol caught more *S. calcitrans* in field trials. In the context of controlling disease vectors, oviposition-site semiochemicals acting as an attractant for gravid female insect are expected to be most efficient because they lure physiologically older females that have already taken a blood meal at least once and therefore are more likely to be infested by pathogens (Marayati et al. 2015). Therefore, by targeting gravid females, control efforts can simultaneously reduce pathogen transmission and control population growth. Also, such an attractant has the advantage of being specific, non-toxic, and effective at very low concentration (Reisenman et al. 2016). However, the semiochemicals that gravid female *S. calcitrans* use to locate suitable oviposition substrates are not yet defined.

1.5 Host finding

Stomoxys calcitrans feeds on a wide range of animals, including rats, guinea pigs, rabbits, monkeys, horses, humans, camels, goats, pelicans, and cattle (Lehane 2005; Pitzer et al. 2011). Adults are diurnal, typically biting during the late morning or earlier afternoon, which indicates that visual cues may contribute to their host-seeking behaviours. To find a suitable host for a blood meal, *S. calcitrans* use olfactory cues (Cilek 1999; Birkett et al. 2004). Jeanbourquin (2005) and Tangtrakulwanich et al. (2011) have shown that various host-associated volatile compounds elicit electrophysiological and behavioural responses from adult male and female *S. calcitrans*. As well as olfactory cues, the host choice for feeding in *S. calcitrans* is also influenced by several factors including behavioural, physiological, morphological, ecological, geographical, temporal and genetic considerations. For example, *S. calcitrans* mostly feed on the lower parts of a cow′s front legs, where shorter hairs present less of an obstacle (Foil et al. 1994). When it comes to making a choice, *S. calcitrans* prefer to feed on camel blood (Higgins 1986); with two peaks of activity: 08:00 am to 10:00 am and 4:00 pm to 6:00 pm (Masmeatathip et al. 2006; Zhu et al. 2016). These flies have a great capacity to cover long distances when searching for blood meals. Taylor et al. (2010) found newly emerged *S. calcitrans* individuals 5 km away from their developmental site. Foil and Hogsette (1994) found flies 100 km from where they were marked, and even up to 225 km after a storm.

1.6 Oviposition site selection

The choice of oviposition site is important for both survival and the population dynamics of insects (Khaliq et al. 2015). In holometabolous insects, oviposition behaviour is critical for larval survival since they have relatively little mobility and depend on the nutritive resources of the substrate selected by adult females. As the preference-performance hypothesis predicts, to ensure the survival of their offspring, gravid females of insects should select substrates that will enhance their fitness (Jaenike 1978). The acceptance or rejection of a particular substrate for oviposition is governed by several factors including visual cues, olfactory cues, and physicochemical properties of the substrate (Bentley and Day 1989; Resetarits Jr 1996).

Flight towards a putative breeding site is mostly controlled by the visual and olfactory cues (considered to be long-range), which allow gravid females to identify different habitats and oviposition site characteristics (Bidlingmayer 1994). The role of visual cues in oviposition site selection is documented in different insect groups. In *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) for instance, Panigrahi et al. (2014) found that either in the light or dark conditions, gravid females of both species laid more eggs on black ovistrip compared to those coloured red, yellow, green, blue and white. Similarly, gravid females of the parasitic beetle *Dastarcus helophoroides* (Coleoptera: Bothrideridae) prefer substrates coloured black over those coloured red, yellow, green and orange (Lyu et al. 2018). Gravid females of the blow fly, under lights, *Chrysomya rufifacies* (Diptera: Calliphoridae) lay significantly more eggs on pork liver with their conspecific larvae in comparison with liver without larvae. But in the dark, these female flies lay the same amount of eggs on both substrates. This suggests that *C. rufifacies* use visual cues to detect the presence of their conspecific larvae on the substrate (Yang and Shiao 2012).

Chemical cues (odour plumes) assist gravid females in orientation towards and landing on oviposition substrates. These cues originate from various sources including substrate, conspecific and allospecific immature stages, and the microbial community of the substrate (Himeidan et al. 2013; Afify and Galizia 2015). For instance, in *Culex quinquefasciatus* (Diptera: Culicidae), oviposition in fermented Bermuda grass (*Cynodon dactylon* (Poales: Poaceae)) is guided by 10 compounds (3-methylindole (skatole), p-cresol, nonanal, 2-undecanone, 2-tridecanone, naphthalene, dimethyltrisulfide, 4-ethylphenol, indole, and phenol) arising from this substrate (Du and Millar 1999). In the same species, the pheromone (*5R,6S*)-6-acetoxy-5-hexadecanolide from the apical droplets of conspecific egg-rafts is

known to attract gravid females (Laurence and Pickett 1985). The dodecanoic, hexadecanoic and tetradecanoic acid isolated from *Ae. aegypti* eggs possess remarkable oviposition attractancy to *Cx. quinquefasciatus* (Sivakumar et al., 2011). Silberbush et al. (2010) demonstrated that the common mosquito predator, *Notonecta maculata* (Hemiptera: Notonectidae), releases two hydrocarbons n-heneicosane and n-tricosane which repel gravid females of *Culiseta longiareolata* (Diptera: Culicidae). Lindh et al. (2008) identified a bacterial-derived volatile, 3-methyl-1-butanol as a putative oviposition attractant of *Ae. aegypti* gravid females.

After landing on a substrate and before egg expulsion, Yang et al. (2008) explained that females stereotypically bend their abdomen and extrude the ovipositor into the substrate. The possible role of the ovipositor is to monitor the quality on the substrate (e.g., temperature, moisture, nutritional value, etc.) before eggs are expelled (Stoffolano and Yin 1987). Several studies have illustrated the effect of substrate physiochemical parameters on oviposition decisions of various insect groups. Notter-Hausmann and Dorn (2010) showed that gravid females of the oriental fruit moth, *Grapholita molesta* (Lepidoptera: Tortricidae), preferred substrates of 30°C over substrates of 22 and 25°C. *Drosophila melanogaster* and *Drosophila biarmipes* (Diptera: Drosophilidae) strongly prefer softer substrates for oviposition (Karageorgi et al. 2017). In *D. melanogaster*, Schwartz et al. (2012) demonstrated that egg-laying decisions are related to the size and the sugar content of the substrate. The authors found that with larger experimental substrates, females preferred to lay directly on substrates with sugar over other substrate types (plain, bitter or salty). Conversely, with smaller substrates, females avoided sweet substrates and laid on non-sweet substrates. Additionally, in the dual test, when the authors offered the choice to flies of laying on a sweet (sucrose) or bitter substrate (caffeine), they preferred the substrate with sucrose. Similarly, when given a choice between a sweet substrate and salty substrate (NaCl), they preferred the sweet substrate. In *Ae. aegypti* and *Ae. albopictus* the number of eggs laid is positively correlated with the ovitrap diameter and negatively correlated with NaCl concentration (Panigrahi et al. 2014).

Besides the importance of visual and chemical cues in oviposition site selection, several authors have also pointed out the possible implication of the pre-imaginal stages experience in oviposition site selection. Two hypotheses support this implication: the "Hopkins' host selection principle" (Hopkins 1916) and the "chemical legacy hypothesis"(Corbet 1985). The

Hopkins' host selection principle (HHSP) proposes that gravid female insects prefer to lay on the substrate on which they developed. The chemical legacy hypothesis suggests that the traces of chemical cues (from the rearing substrate) persisting on the adult body after emergence influence oviposition decisions. To illustrate this, McCall and Eaton (2001) reared *Cx. quinquefasciatus* larvae on water with different concentrations of p-cresol and skatole. They found that gravid females that had been reared as larvae in water with highly concentrated p-cresol and skatole laid significantly more eggs in a pool of water containing similarly high concentrations of the two chemicals.

1.7 Chemical ecology

1.7.1 Olfactory system: antenna, sensilla types and odorant receptors

To locate and evaluate food, developmental substrates, shelter, and mates, insects rely on a wide range of sensory systems situated on antennae, mouthparts, wings, legs and ovipositors (Hansson and Stensmyr 2011; Ali et al. 2015). Among these organs, antennae are the primary sensory structure for detecting volatile chemical cues in insects (Olsson and Hansson 2013). In Diptera, three types of antennae are mainly found: (1) antennae with three segments and aristae (Fig. 1.5A1-A3; characteristic of Cyclorrhapha group where *Stomoxys calcitrans* are found (Fig. 1.5A2)), (2) antennae with five or fewer segments (Fig. 1.5A4-A6; found in Orthorrapha groups such as tabanids) and (3) antennae with more than six segments (Fig. 1.5A7-A9, mostly found in Nematocera groups such as mosquitoes). As in all insects, the antennae of Diptera comprise three sections: the basal "scapus", fixed to the head capsule, the "pedicellus", housing the Johnston's organ, and the "flagellum" cover with the hair-like structures called sensilla (Huotari 2004; Hansson and Stensmyr 2011; Guidobaldi et al. 2014) (Fig. 1.5B). The "flagellum" is considered a purely olfactory organ; sensilla located on the surface of antenna house one or more olfactory sensory neurons (OSNs) that expresses odorant receptors to detect volatile odours (Onagbola and Fadamiro 2008). The numbers of sensilla and OSNs per antenna sometimes vary among species and between sexes. For example, the antenna of *Manduca sexta* contains more than 100,000 sensilla housing more than 250,000 OSNs (Sanes and Hildebrand 1976), whereas the *D. melanogaster* antenna has around 400 sensilla housing approximately 1200 OSNs (Shanbhag et al. 1999). In *Ae. aegypti*, female antennae possess three to four times more sensilla than male antennae (McIver 1982). It is also possible to find sensilla in other parts of insect body including

mouthparts (labial and maxillary palps, and proboscis), the ovipositor, legs, and wings (Silbering and Benton 2010). For example, the maxillary palps of *An. gambiae* (Lu et al. 2007), the labial palps (Guerenstein et al. 2004) and the ovipositor (Klinner et al., 2016) of *M. sexta* house sensilla. Depending on their external morphology (length and pore density), the sensilla can be classified into different types: trichodea, placodea, basiconica, chaetica, coeloconica, styloconica, and campaniformia (Pellegrino 2011; Seada 2015). Tangtrakulwanich et al. (2011) have shown that the *S. calcitrans* antenna has four sensillar types: basiconica (Fig. 1.5C1), clavate (Fig. 1.5C2), coeloconica (Fig. 1.5C3) and trichoid sensilla (Fig. C4). Sensilla can also be categorised based on their putative function, on which some are chemoreceptors (e.g. basiconica and trichoidea), mechanoreceptors (e.g. chaetica), thermoreceptors (e.g. coeloconica) and hygroreceptors (e.g. digitiformia) (Ali et al. 2015, Grabe and Sachse 2018). In addition to OSNs, sensilla also house auxiliary cells called thecogen, trichogen and tormogen cell (Keil 1999); with the two later providing ions and proteins to the sensillum lymph (Guidobaldi et al. 2014)**.**

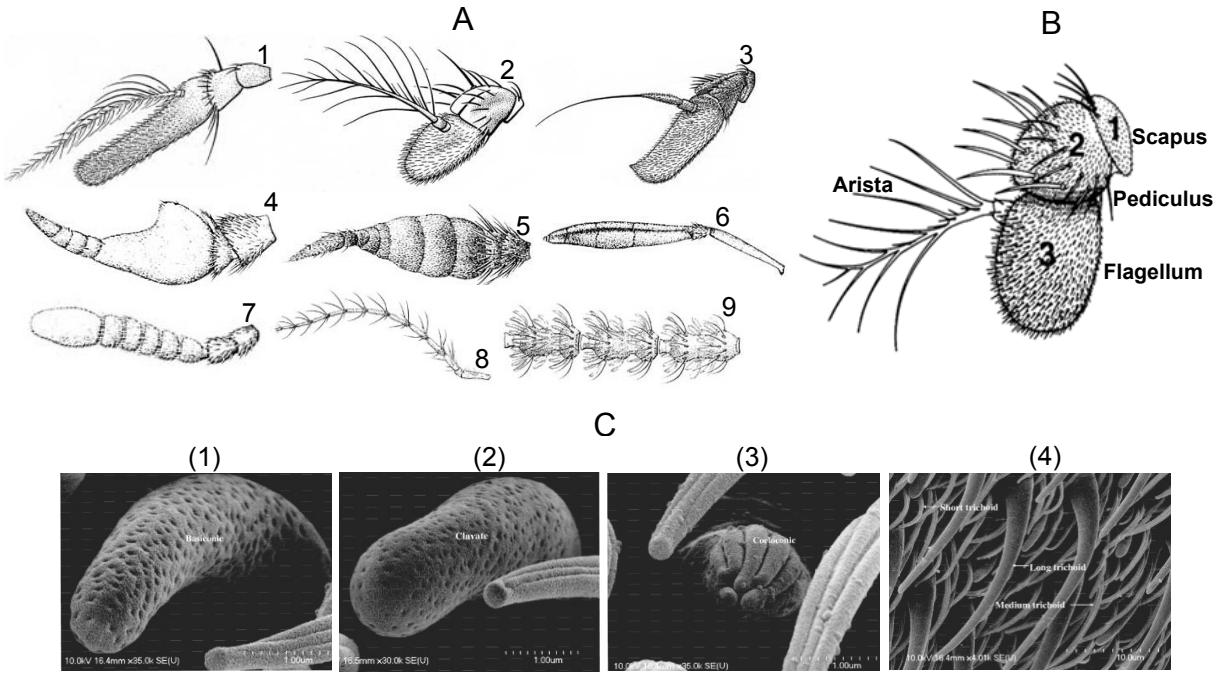

Figure 1.5. Organisation of Diptera antenna. (A) External shape of Cyclorrhapha (1), Orthorrhapha (2) and Nematocera (3) antenna (source: phorid.net). (B) Morphological parts of Diptera antennae (Source: Eberl et al. (2000)). (C) Scanning electron microscopy (SEM) of *S. calcitrans* sensillum types: basiconic (1), clavate (2), coeloconic (3), and trichoid (4) (Source: Tangtrakulwanich et al. (2011)).

1.7.2 Chemoreceptor function

- Physiology

Insect sensilla (Fig. 1.6A) are the structural and functional unit of the antenna through which the nervous system interacts with the external world. The perception of an ecologically relevant odour starts when the odour molecule is intercepted by an olfactory sensillum (Fig. 1.6B). It then passes through a sensillum pore and reaches the receptor lymph where it is transported by an odorant binding protein (OBP) to the dendritic membrane of an olfactory sensory neuron (OSN) where stimulus transduction may occur (Tsuchihara et al. 2005). Signal transduction is initiated by the odour binding to its receptor, which then triggers a cascade of events inside the dendrite leading to nerve cell activity that results in an electrical signal referred to as an action potential (Leal 2004; Pelosi et al. 2006) (Fig. 1.6 C). There is still a controversial discussion of how odour signals are transduced into the electrical signal. Some authors support the direct odour-gated fast ionotropic hypothesis suggesting that once an odour molecule binds to the complex odorant receptor (OR)-odorant coreceptor (Orco), the odour molecule initiates a conformational change in the OR that opens a channel in its structure. This leads to an influx of cations (Ca^{2+}, K^{+} and Na^{+}) into the neuron that generates a fast ionotropic current due to a rapid depolarisation of the neuron membrane (Nakagawa et al. 2005; Sato et al. 2008). Others support the metabotropic hypothesis postulating that the liaison between the odour and the OR-Orco triggers a cascade of reaction that involve the activation of the G-protein and adenylyl cyclase enzyme that leads to the production of the second messenger cyclic adenosine monophosphate (cAMP) which indirectly leads to depolarisation the neuron membrane by activating ion channels separate from the OR (Wicher et al. 2008; Stengl and Funk 2013; Getahun et al. 2016). The action potential from the transduction (Fig. 1.6D) goes through the OSN axon and reaches the primary olfactory centre, the antennal lobe (AL) formed by spherical structures called glomeruli (Meyer et al. 2013). This structure is usually innervated by axons from OSNs that express the same type of ORs (Vosshall et al. 2000; Couto et al. 2005). Overall, most Diptera species have 50 - 70 glomeruli; a number that is species- and sex-specific. Lin et al. (2018) described 64 - 65 identifiable glomeruli in both sexes in *Bactrocera dorsalis*. In *Ceratitis capitata*, Solari et al. (2016) numbered 53 glomeruli. Sexual dimorphism in the AL is also observed in other Diptera. The AL of female *Ae. aegypti* contains 51 glomeruli whereas that of male comprises 49 glomeruli (Ignell et al. 2005). In *A. gambiae*, the AL of males and females have 61 and 60 glomeruli respectively (Ghaninia et al. 2007). When it reaches the glomeruli, the actional

potential is transferred from the OSN axons (via neurotransmitters) to the dendrites of projection neurons (PNs) that transmit the processed signal to the higher brain, namely the mushroom body and the lateral horn (Vosshall and Stocker 2007), leading to translation into a behavioural output such as acceptance or avoidance of oviposition site, food, or sexual partner (Kreher et al. 2008). Interestingly, feeding and oviposition activate distinct groups of glomeruli in the antennal lobe showing the two are different circuits (Bisch-Knaden et al. 2018).

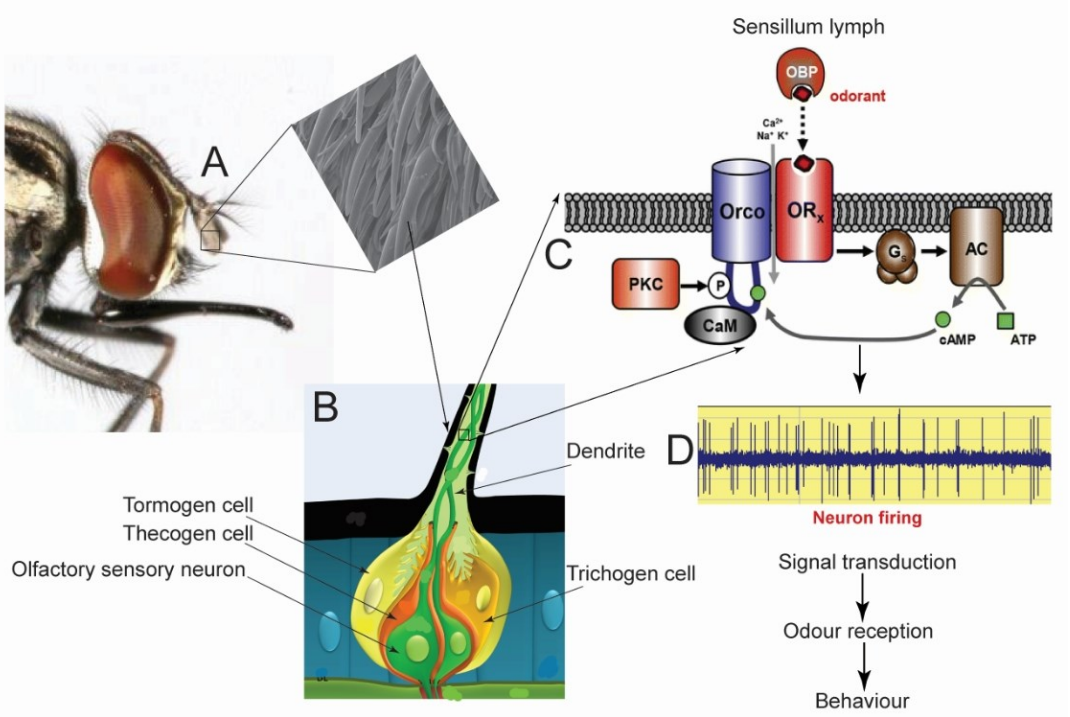

Figure 1.6. Schematic representation of insect olfaction physiology. (A) *Stomoxys calcitrans* sensilla density (head and sensilla are original photographs), (B) Anatomy of trichoid sensilla (modified from diagram of Stengl 2010), (B) Ultrastructure of olfactory sensory neuron membrane (from Fleischer et al. 2018); (D) Spike amplitude depicting action potential in *S. calcitrans* (Original photo).

- Techniques used to identify behaviourally active compounds

Electrophysiology is an important method to rapidly quantify the response of an insect to odour stimuli. Olsson and Hansson (2013) described three methods: electroantennography (EAG), gas chromatography coupled with electroantennographic detection (GC-EAD), and single sensillum recording (SSR).

EAGs are performed by recording the electrical changes that occur between the distal and the proximal part of the antennal flagellum due to semiochemical stimulation. These

electrical changes correspond to the action potentials of several olfactory neurons activated by a chemical stimulus (Roelofs 1984). The development of GC-EAD was important in the field of analytical chemistry because it uses an insect antenna as a sensing element to identify specific volatile organic compounds (VOCs) from complex odour mixtures as they elute from a GC capillary column (Arn et al. 1975). Several studies using EAG and GC-EAD have already been conducted in *Stomoxys calcitrans*. Schofield et al. (1995) through the EAG method, found that 1-octen-3-ol elicited the strongest electrical activity in *S. calcitrans* antenna compared to the other synthetic chemicals known to be electrophysiologically-active for other biting flies (pentan-1-ol, hexan-1-ol, heptan-1-ol, etc…). Using EAG, Tangtrakulwanich et al. (2011) studied the olfactory response of *S. calcitrans* to the host-associated odorant compounds. Also, Hieu et al. (2014) through EAG assessed the effect of a mixture between attractants and repellents in *S. calcitrans* antennae response. Based on the GC-EAD technique, Jeanbourquin and Guerin (2007) have elucidated the antennal response of *S. calcitrans* to the odour emanating from cow, horse dung and to the rumen extract odour.

The EAG signal is a combined response of many types of receptors including receptors for mechanosensitive sensilla responding to air turbulence (Pers and Minks 1993). Also, this signal relies on insect vitality, and the position and the strength of electrode connection (Nagai 1983; Crnjar et al. 1989). In contrast, "single unit" or SSR measures the electrical activity by a single sensillum records action potentials elicited by olfactory neurons within a single sensillum after its stimulation by an odour (Schneider and Boeckh, 1962). SSR can be used to identify the number of olfactory neurons (by comparing the spike amplitude) housed in a specific sensillum (Ghaninia et al. 2008). Moreover, with SSR an excitatory stimulation (increased spike frequency) can be differentiated from an inhibitory stimulation (spike cessation) (de Bruyne et al. 1999; Diehl et al. 2003).

The electrophysiological techniques presented above are expensive, tedious and time-consuming. They involve multiple rounds of separation and testing until the identification of chemical cue(s) that mediate targeted behaviour. To alleviate this workload, Dam & Poppy (2008) suggested the used of machine learning algorithms in the field of chemical ecology. The authors explained that among the multitude of volatiles that are present in the environment, insects can identify specific odours that are released from a target source (e.g. oviposition sites, sexual partners, hosts, etc…); a property which is very similar to the dimension reduction and feature selections implemented in the field of machine learning. For Dam & Poppy (2008), chemical ecologists need to adopt methods from bioinformatics

that employ accurate methods of classifying volatile organic compounds (VOCs). These include machine learning methods such as principal component analysis (PCA), discriminant analysis (DA), support vector machines (SVM), and random forest analysis. Several examples are found in the literature where researchers used these techniques to identify behaviourally-active candidate VOCs. Scheidler et al. (2015) used PCA analysis to identify odourants that are most influential in *Drosophila* yeast discrimination. Using the same technique, Giunti et al. (2018) isolated six promising attractants and six repellents for *Tribolium confusum* (Coleoptera: Tenebrionidae), a secondary pest of stored cereals. Also, Darshanee et al. (2017) found chemical volatiles that may attract or repel the whitefly *Trialeurodes vaporariorum* (Hemiptera: Aleyrodidae) based on PCA analysis. Tauxe et al. (2013) identified new candidate attractants and repellents for mosquitoes by screening > 400.000 chemical compounds using the SVM analysis.

The random forest analysis which has been performed in this thesis (Chapter 1) is widely implemented by chemical ecologists. Marneweck et al. (2017) used this analysis to elucidate the chemical signals that white rhinos, *Ceratotherium simum* (Perissodactyla: Rhinocerotidae) use to discriminate the sex, age, territorial and oestrous state of their conspecifics. Also, based on the volatiles emanating from the dung of *C. simum*, the same authors used the random forest analysis to distinguish the dung age of this vertebrate between the dry and rainy season (Marneweck et al. 2017b). In the gypsy moth, *Lymantria dispar* (Lepidoptera: Erebidae), McCormick et al. (2016) used this analysis to determine the volatile that the caterpillar of this moth used to distinguish damaged leaves from undamaged leaves of the black poplar tree *Populus nigra* (Malpighiales: Salicaceae). Mansourian et al. (2016) identified chemical volatiles that *Drosophila* used to differentiate carnivore from herbivore dung based on the random forest technique. Based on the random forest approach, De Moraes et al. (2014) elucidated the chemical volatiles used by *Aedes stephensis* to distinguish *Plasmodium chabaudi*-infected mice from healthy mice. Ranganathan and Borges (2010) specified that, as compared to other classification algorithms, random forest analysis is best suited for volatile analyses: (i) it allows for more volatiles than samples; (ii) it has a good classification efficiency, even with a lot of background noise; (iii) it is capable of arriving at a minimal set of volatiles, which can be used as predictors of that particular samples; (iv) it is robust to interactions and correlations among volatiles; (v) it gives measures of relative volatile importance.

1.8 Thesis rationale

In *Stomoxys calcitrans*, all kinds of plant decaying matter such as rotting hay, silage, grass clippings, and garden compost may form an appropriate substrate for breeding (Zumpt 1973, Cook et al. 2018). *S. calcitrans* also oviposit on wild and domestic vertebrate herbivore dung (Mavoungou et al. 2017). Most studies on *S. calcitrans* oviposition preference focus on screening domestic herbivore dung, neglecting dung from wild herbivore animals (zebra, giraffe, elephant, etc...), which are also present in the environment of *S. calcitrans*. For instance, Hafez and Gamal-Eddin (1959) reported that *S. calcitrans* prefer to oviposit on equine dung (donkey and horse) than on cattle dung. Jeanbourquin and Guerin (2007) have shown that when exposed to horse and cow dung, *S. calcitrans* preferred to lay on horse dung. Machtinger et al. (2014) further demonstrated that this preference for equine dung was positively related to the greatest development of *S. calcitrans* larvae. However, these studies did not examine the olfactory cue(s) governing the observed preference behaviour. Even though Jeanbourquin and Guerin (2007) identified the chemo-stimulants that elicit electrical responses by *S. calcitrans* antenna, the authors did not further demonstrate their ability to trigger a behavioural response (oviposition) in *S. calcitrans* gravid females. Even semiochemicals (phenol, m-cresol and p-cresol) isolated from cattle manure slurry by Tangtrakulwanich et al. (2015) did not show specificity in attracting gravid females of *S. calcitrans*. In addition to this scarcity of information about the specific olfactory cue(s) triggering oviposition decision in gravid females of *S. calcitrans*, the previous authors did not test the possible influence of biotic interactions (intra and interspecific competition, parasitism) and larval experience on oviposition decision of *S. calcitrans* gravid females.

1.9 Thesis aim and objectives

The aim of this thesis was to examine the oviposition decisions of gravid female stable flies, *Stomoxys. calcitrans* L. (Diptera: Muscidae), and the associated fitness consequences for offspring. To achieve this aim I established and tested the following four hypotheses, each of which is addressed in separate chapters:

- Oviposition site selection in gravid females of *S. calcitrans* is related to the performance of their pre-imaginal stages and is driven by olfactory cues.
- *Stomoxys calcitrans* avoid ovipositing on substrates occupied by conspecific larvae, heterospecific larvae or parasites to avoid intra- and interspecific competition or parasitism by their progeny.
- Larval density and substrate quality affect wing size and shape of *S. calcitrans*.

- Larval experience can affect oviposition decisions in gravid female *S. calcitrans*.

CHAPTER 2 Egg-laying decisions based on olfactory cues enhance offspring fitness in *Stomoxys calcitrans* L. (Diptera: Muscidae)

This chapter is published as:

Baleba, S. B. S., Torto, B., Masiga, D., Weldon, C. W. and Getahun, M. N. (2019). Egg-laying decisions based on olfactory cues enhance offspring fitness in *Stomoxys calcitrans* L. (Diptera: Muscidae). *Scientific Reports* **9**: 3850.

2.1 ABSTRACT

Selection of oviposition-substrate is critical in holometabolous insects. Female stable flies, *Stomoxys calcitrans*, locate and select vertebrate herbivore dung in which they lay their eggs. However, the preference for vertebrate herbivore dung by *S. calcitrans* females, its fitness consequences for offspring, and the semiochemicals used to locate and select oviposition substrates remain unclear. Using oviposition choice tests and life table bioassays I found that gravid female *S. calcitrans* prefer to oviposit on donkey and sheep dung, which also improves the performance of their offspring. GC-MS analysis followed by random forest classification identified β-citronellene and carvone as the most important predictive volatile organic compounds of donkey and sheep dung, respectively. In multiple choice oviposition bioassays, *S. calcitrans* laid more eggs in wet sand containing β-citronellene and carvone than in other treatments. The attractiveness of these compounds was confirmed in a field trial, with traps baited with β-citronellene and carvone catching more *S. calcitrans*. I conclude that gravid female *S. calcitrans* use semiochemical cues to choose oviposition substrates that maximise offspring fitness.

2.2 INTRODUCTION

In holometabolous insects, oviposition site selection by gravid females plays an important role in their distribution, abundance, and population dynamics owing to the immobility of egg stages combined with the lack of parental care (Deas and Hunter 2012) . Location and selection of the most appropriate substrate for oviposition involves visual, olfactory and mechanical cues (Bentley and Day 1989). According to Städler (1994) , the nutritional and chemical composition of the environment determine the success of development in almost all insects, thus olfactory cues play a paramount role during oviposition site selection. When selecting a suitable site for oviposition, insects might use a single chemical cue (Dweck et al. 2013; Guidobaldi and Guerenstein 2015) or a mixture of important chemical cues (Späthe et al. 2013; Riffell et al. 2014; Lindh et al. 2015; Wondwosen et al. 2016).

Jaenike (1978) postulated that gravid female insects prefer to oviposit on substrate that maximise the fitness of their offspring. This was termed the 'preference-performance' or 'mother knows best' hypothesis. Oviposition substrates used by female insects include animal dung, fruits, and leaves which are abundant and rich in nutrients. The preference-performance relationship has been widely explored in phytophagous insects, with some studies confirming a positive relationship between preference and performance, while others show poor correspondence (Craig et al. 1989; Valladares and Lawton 1991; Ladner and Altizer 2005a; Clark et al. 2011; Gómez Jiménez et al. 2014). In hematophagous insects, understanding of the preference-performance relationship is most advanced in mosquitoes. For instance, there is a positive correlation between oviposition preference and larval performance in *Culiseta longiareolata* (Kiflawi et al. 2003), *Aedes triseriatus*, and *Aedes albopictus* (Reiskind et al. 2009). whereas in *Wyeomyia smithii* (Heard 1994) and *Aedes aegypti* (Wong et al. 2012), a negative correlation has been observed. However, these studies do not consider the chemical basis driving the preference-performance interaction. Here I investigated the preference-performance hypothesis in the stable fly, *Stomoxys calcitrans* (Diptera: Muscidae), and the chemical basis involved in this interaction.

Stomoxys calcitrans is a cosmopolitan blood-feeding insect of medical and veterinary importance (Dsouli-Aymes et al. 2011). For the success of their mating, egg production and survival, both female and male *S. calcitrans* depend on repeated blood meals from diverse domestic (e.g., camel, cattle, horse) and wild animal hosts (e.g., buffalo, antelope, zebra) (Mihok et al. 1996; Broce and Haas 1999) as well as humans (Yeruhan and Braverman

1995). During their blood meals, *S. calcitrans* can transmit viruses (e.g., West Nile fever virus) (Johnson 2010), bacteria (e.g. *Bacillus anthracis*) (Hugh-Jones and Blackburn 2009), protozoans (eg. *Trypanosoma evansi)* (Sumba 1999) and helminths (e.g. *Habronema microstoma)* (Traversa 2007). In the USA alone, Taylor et al (2012) estimated that economic losses attributed to *S. calcitrans* infestation were >$2 billion per year.

The dung of vertebrate herbivore animals is used as an oviposition substrate by female *S. calcitrans* (Hafez and Gamal-Eddin 1959; Jeanbourquin and Guerin 2007; Cançado et al. 2013). However, preference by *S. calcitrans* for vertebrate herbivore dung of different species, the fitness costs to its offspring, and the semiochemicals involved remain unclear. In this study, I investigated the preference-performance hypothesis in *S. calcitrans* oviposition behaviour on dung of different vertebrate herbivores, and the semiochemical basis of this interaction. I demonstrate that gravid female stable flies oviposit on substrates that have fitness benefits for their offspring using finely tuned responses to semiochemical cues in preferred substrates such as β- citronellene and carvone. I then used these semiochemicals to perform laboratory and field trials to test their attractiveness to female *S. calcitrans.* This information will prove useful for developing effective lures to attract and kill gravid females, and thereby suppress *S. calcitrans* abundance.

2.3 MATERIAL AND METHODS

Insects and oviposition substrates

To establish the colony, wild individuals of *S. calcitrans* were captured at *icipe* Duduville campus in Nairobi (1˚13'12" S, 36˚52'48" E; 1,600 m above sea level) using a Vavoua trap. Trapped adults were transferred to cages (75 × 60 × 45 cm) in an insectary maintained at 25 ± 5˚C and 65 ± 5% relative humidity, with a 12L: 12D photocycle. Flies were fed two times per day (0800 and 1600 hours) on defibrinated bovine blood on moistened cotton. The rearing medium consisted of rabbit dung. Rabbit dung was placed in plastic containers (21.5 × 14.5 × 7.4 cm) that were introduced to the adult cage for two days to allow oviposition. Afterwards, the rearing medium was removed and transferred to another cage and followed daily from egg hatch to the pupal stage. Pupae were placed in Petri dishes, removed from the cage, and introduced to another cage for age-matched adult emergence. As described above, blood and rearing media were provided to the newly emerged adults to obtain flies for experiments.

For our oviposition bioassay, I screened fresh dung of buffalo, camel, cow, donkey, elephant, giraffe, sheep, and zebra, which are potential breeding sites for *S. calcitrans* and abundant in the region where the study was conducted. The dung types were collected from different agroecological zones, including Kapiti Plain in Machiakos County (1˚37'60"S, 37˚0'0"E), Ngurunit in Marsabit County (1˚59'58" S, 37˚30'11" E), and Shimba Hills located in Kwale County (04˚15'26''S, 39˚23'16''E), Kenya. These localities are characterised by the presence of several populations of wild and domestic vertebrate herbivores, as well as biting flies such as *S. calcitrans*. Dung was collected immediately after it was deposited by each species (within 24 hours). To our knowledge, none of the vertebrate herbivore populations from which dung was collected received anthelmintic treatments that could potentially affect development of coprophagous insects (Sommer et al. 1992).

Preference-performance hypothesis test

Multiple-choice oviposition preference bioassay with vertebrate herbivore dung

To assess oviposition preference of gravid female *S. calcitrans*, I began with a multiple-choice oviposition bioassay in semi-field conditions, using wild, gravid female, easily recognized by examining the ventral abdomen filled with eggs. *S. calcitrans* caught directly from the field. A cage (75 × 60 × 45 cm) containing 30 wild gravid females was placed outside in a shaded, sheltered location. Fresh dung (60 g) from buffalo, camel, cow, donkey, elephant, giraffe, sheep, and zebra, as well as grass and wet sand (control), was placed in Petri dishes and introduced to the cage. Each Petri dish was separated by at least 20 cm in a circle, with the order of dung being haphazard. Twenty-four hours after setting up the bioassay, the total number of eggs laid on each substrate was counted. The cages were placed outside at the *icipe* Nairobi campus from May to June 2017, during which 30 replicates were performed. Mean temperature during this period was 22.5 ± 4.7˚C, with 55% relative humidity, and 12L: 12D photoperiod.

To check the validity of the result from field collected flies under semi-field conditions, I conducted the same experiment in a controlled laboratory environment (at 25 ± 5˚C and 65 ± 5% relative humidity, with a 12L:12D photocycle) using 30 naïve, gravid females *S. calcitrans* (aged from 4 to 6 days) from our established culture. Here, the oviposition preference was assessed after 24 hours by recording three parameters: (1) number of batches

deposited on each substrate, (2) number of eggs per batch, and (3) the total number of eggs deposited on each substrate. The laboratory experiment was replicated ten times.

Performance test

I conducted an incomplete cohort life table study for the immature stages of *S. calcitrans* using camel, cow, donkey and sheep dung. This was to test if a detected preference of gravid female *S. calcitrans* to oviposit on donkey and sheep dung in comparison with camel and cow dung was related to the performance of their offspring. Ten *S. calcitrans* eggs were artificially introduced to each type of dung (n = 10) and followed daily until adulthood to record six parameters: (1) egg hatchability, (2) larval development time (from egg to pupa), (3) pupal development time (from pupa to adult eclosion), (4) larval weight, (5) larval growth rate and (6) pupal weight. Eggs for each treatment were obtained by placing the same dung type in the established culture cage 24 hours before commencing the test. The weight parameter was recorded individually on 40 larvae and pupae coming from each substrate. Larval weight was recorded 5 and 10 days after egg hatch. Larval growth rate was calculated as (Ladner and Altizer 2005) $[M_{day10}-M_{day5}]/t$, where M_{day10} was the larval mass at day ten, and M_{day5} was the larval mass at day five, and t, was the number of days intervening between the two consecutive weight measurements.

Dung sample physico-chemical characterisation

To elucidate whether the performance of *S. cacitrans* offspring is related to the chemical composition of their substrate of development, I determined the proportion of nitrogen (N), carbon (C), pH, and micronutrients [copper (Cu), phosphorus (P), potassium (K), zinc (Zn)] present in camel, cow, donkey and sheep dung using lyophilised (freeze-dried), ground samples (n =10). The total N was determined using the Kjeldahl method with 0.5 g of each dung material (Vilanova et al. 2010). The total C expressed as a percentage of residues was determined after 4 hours of ignition at 500˚C in a muffle furnace using a 0.5 g sample of each dung (Matthiessen et al. 2005). The pH was measured using the potentiometric method after water sample extraction (by shaking 1:2 w/v of each sample for 20 minutes at 180 rpm). The micronutrients were measured from 0.5 g of each sample using the atomic emission spectrometry (ICP-OES) method following the microwave digestion procedure with nitric acid and hydrochloric acid (Boss and Fredeen 2004). Additionally, to determine the water content of each animal dung, I recorded the volume (V) and weight (W_{wet}) of ten fresh samples. Afterwards, I determined the dry weight (W_{dry}) of the same samples by placing

them in an oven at 100˚C and reweighing them several times until constant weight. I calculated the water content, and the proportion of dried matter of each animal dung using the formula (Bremner 1996) $(W_{wet}-W_{dry})/ W_{dry}$; and $(W_{wet}-W_{dry})/V$.

Characterisation of semiochemicals emanating from oviposition substrates

Odour collection

Odours were collected from fresh dung samples from buffalo, camel, cow, donkey, elephant, giraffe, sheep, and zebra using a dynamic headspace apparatus(Tholl et al. 2006). In this setup, ambient air was passed through copper tubing to activated charcoal (for purification), then into a bubble humidifier containing double distilled water. The humidified air was supplied by a vacuum at a flow rate of 150 ml air min^{-1} through multiple ports (manifolds) to the dung samples enclosed in glass jars, which were connected in parallel. Each glass jar had a port for a Super Q adsorbent, which trapped any volatile organic compounds from the vacuum air stream. For our collection, 500 g of each dung type (replicated five times) was introduced and sealed inside the glass jars (oven sterilised at 100°C for 24 hours). Before use, each adsorbent was cleaned ten times with 200 µL hexane, followed by dichloromethane to avoid contamination. Volatiles were collected for 24 hours. Trapped volatile compounds were eluted by washing the adsorbent with 600 µl hexane and stored in air-tight glass vials at -20˚C until analysed by gas chromatography linked with mass spectrometry.

Gas chromatography linked with mass spectrometry

The collected volatile compounds were analysed using a gas chromatograph coupled to a mass spectrometer (GC–MS; HP 6890 GC and 5975 MS; Agilent Technologies, Palo Alto, CA, USA) in the electron impact ionisation mode at 70 eV. Each sample (1 µL) was injected into the GC-MS with an autosampler (Agilent Technologies). Injections of the volatile extracts were conducted with a splitless injector at 220 °C. Compounds were separated on a nonpolar capillary HP column with helium as the carrier gas at an average linear flow rate of 35 cm s^{-1}. The oven temperature was held at 35°C for 5 min and then increased by 10°C/min to a final temperature of 280°C, which was held for 10 min. Volatiles were then identified by comparison of their mass spectra and retention times with those of commercial standards and library database spectra using the NIST mass spectral search program (ver. 2.0), Pherobase (http://www.pherobase.com) and the NIST web book

(http://webbook.nist.gov/chemistry). The identified chemicals were confirmed by co-injection with authentic standards and comparison with both the expected retention time and MS spectra.

Volatile chemical classification

To identify the volatiles that are abundant and permanently present across all the replicates of each herbivorous vertebrate dung (most important volatiles), I performed random forest (RF) analysis using the relative abundance of each identified volatile organic compound (Breiman 2001) The RF analysis is a mathematical algorithm that uses results from several decisions trees to classify a large number of variables (chemical volatiles in our case) (Ranganathan and Borges 2010). Compared to other classification methods such as principal component analysis (PCA), the RF analysis is advised for volatile importance classification. This is because (1) it allows for more variables; (2) it has a good classification efficiency; (3) it is capable of arriving at a minimal set of variables that can be used as predictors for a particular group; (4) it is robust to interactions and correlations among variables; (5) it gives measures of relative variable importance; and (6) it can also be used to analyse time series data that record patterns in volatile emissions over time(Liaw and Wiener 2002; Ranganathan and Borges 2010; Marneweck et al. 2017)

Multiple-choice oviposition bioassay with the most important dung volatiles

The RF analysis identified carvone as the most important volatile of buffalo and sheep dung. On the other hand, *p*-cymene, limonene, β-citronellene, cyperene, *m*-cresol, and camphene were identified as the most important volatiles of camel, cow, donkey, elephant, giraffe, and zebra dung, respectively. Subsequently, I purchased the synthetic standards of the following compounds: (R)-(-)-carvone (98%), *p*-cymene (99%), (R)-(+)-limonene (93%), (+)-β-citronellene (analytical standard), *m*-cresol (98%), and camphene (95%) (Sigma-Aldrich Germany). Cyperene was not tested due to its unavailability of a commercial product.

The attractiveness of each volatile for *S. calcitrans* oviposition was tested using the concentration 10^{-2} v/v diluted in mineral oil. For the bioassay, seven Petri dishes containing wet sand as an oviposition medium were introduced to a cage (75 × 60 × 45 cm). I placed an Eppendorf tube lid loaded with 100 µl of each volatile solution on the wet sand. The control was wet sand with mineral oil. Thereafter, 30 gravid females of *S. calcitrans* were introduced to the cage. After 24 hours the total number of eggs deposited on each medium was counted.

The experiment was replicated 15 times. Wet sand (control) was avoided by gravid females of *S. calcitrans* in our earlier oviposition preference bioassays, so any enhancement of oviposition indicated a stimulant effect on females.

The results of this bioassay revealed that media containing carvone and β-citronellene from sheep and donkey dung, respectively, attracted more gravid females of *S. calcitrans* for oviposition over the other volatiles. To appreciate the attractiveness effect of carvone and β-citronellene in gravid female *S. calcitrans* oviposition, I conducted another oviposition preference bioassay by replacing these two volatiles with β-caryophyllene and *m*-xylene. β-caryophyllene and *m*-xylene were present in donkey and sheep dung respectively but less important. The same methods were used for this bioassay, which was replicated 15 times, and the total number of eggs laid in each Petri dish of wet sand was counted after 24 hours.

Field trapping assay

Finally, I tested the attractiveness of carvone and β-citronellene to *S. calcitrans* (mainly gravid females) under field conditions. To do so, I conducted a field trapping study at Mpala Ranch located in Laikipia County in central Kenya (Figure 5A; 00° 23' 26.98" N, 036° 52 '14.98" E). This region is characterised by semi-arid savannah vegetation in which *S. calcitrans* are associated with wild animals (elephant, zebra, impala, monkey, lion etc.) and domestic animals (mainly camel, cattle, goat and sheep). Vavoua traps (Mihok et al 1996); (Figure 5B) were baited with 2 ml of undiluted synthetic standards of β-citronellene, carvone, and *m*-cresol, which was a positive control, already known to attract *S. calcitrans* (Tangtrakulwanich et al 2015). Blends of volatile chemicals were also tested. These were formulated using the mean decrease accuracy (MDA) value of their chemicals. Blend A comprised carvone + β-citronellene (3:2), Blend B comprised β-citronellene + valencene (1:1), and Blend C comprised carvone + valencene + γ-terpinene (3.5:3.5:3). Each compound or blend was transferred to a 4 ml glass vial. A cotton dental roll (10 × 38 mm; Shanghai Dochem Industries Co. Ltd.) was inserted inside the vial as a dispenser. The vial was closed with a perforated cap (5 holes of 2 mm diameter (Bengtsson et al. 2009) and gently tilted to soak the cotton dispenser (Figure 2.5C). Traps with vials without chemicals were used as a negative control. Each vial was fixed on the pole of each trap with a metal wire, 0.5 m above the ground.

Traps were placed 150 m apart in a Latin square design. Trapping was carried out for 7 days with daily rotation of vials among the traps. Seven traps per lure were set before 09:00h daily

and checked twice per day at 13:00h and 17:00h. *Stomoxys. calcitrans* were identified based on the key of Zumpt (1973). Identified individuals were sorted by sex, blood-feeding status and egg development. Feeding status and egg development were established by gently piercing the abdomen of each captured female with a needle to verify the presence of blood and eggs, respectively. Three parameters were therefore recorded for each trap: (1) the number of males and females of *S. calcitrans* caught, (2) the number of fed flies caught and (3) the number of gravid females caught.

Data analysis

All analyses were performed using R software (R Core Team 2018) (version 3.5.1) and the R Studio graphical user interface (version 1.1.383). The data from the parameters I used to assess oviposition preference of gravid female *S. calcitrans* (number of batches deposited on each substrate, number of eggs per batch and the total number of eggs laid on each substrate) were subjected to the Shapiro-Wilk test of normality and Levene's test of homoscedasticity. Data were not normally distributed, and variances were not homogeneous ($p<0.05$). Therefore, I used the non-parametric Kruskal-Wallis test followed by Dunn's post hoc test with Bonferroni's adjustment (to avoid type I error) to determine whether *S. calcitrans* oviposition differed among the tested substrates(McDonald 2009).

Immature performance parameters were analysed in relation to substrate as the dependent variable. Egg hatchability data were analysed using a generalised linear model (GLM) with binomial distribution due to the binary nature of this parameter (hatched vs unhatched) (Warton and Hui 2011). The number of hatched and unhatched eggs were taken as the response variable. Model significance was detected by analysis of deviance (with the chi-squared test). Tukey's multiple comparisons tests were performed using the package 'lsmeans'(Lenth 2016) to identify differences in egg hatchability among substrates. Larval development and adult emergence time data were normally distributed (Shapiro-Wilk test: $p>0.05$). so I used analysis of variance (ANOVA) followed by Student-Neuman-Keuls (SNK) post hoc multiple comparisons tests using the R software package called 'Agricolae'(de Mendiburu 2017) to the separate means from each substrate. Larval weight and larval growth rate data were analysed using the non-parametric Kruskal-Wallis test followed by post-hoc Dunn's tests due to the non-normal distribution of the data and the

disparity of their variance. For pupal weight, data were normally distributed, so I used ANOVA followed by the SNK post-hoc tests.

To compare the physicochemical composition of camel, cow, donkey, and sheep dung, I performed the multivariate analysis of variance (MANOVA) followed by SNK tests. To establish whether dung constituents were correlated to *S. calcitrans* oviposition preference and the performance of their offspring, I performed principal components analysis (PCA) using two R packages called "FactoMineR" and "Factoextra"(Kassambara 2017).

I classified the chemical volatiles arising from the dung of each vertebrate herbivore, using the R software package "RandomForest"(Breiman 2001), version 4.6-12. To execute the RF analysis, I ran 10000 iterations (ntree) with 11 volatiles randomly selected at each split (mtry= √q, where q is the total number of volatiles (120)). Based on the function '*importance ()"* I generated the mean decrease in accuracy (MDA), which provides an importance score for each volatile. For each herbivore dung, the volatile with the highest MDA value was considered the most important (Mansourian et al. 2016; Marneweck et al. 2017a). To visualise the similarity of herbivore dung volatile composition, I generated a multidimensional scaling (MDS) ordination plot (Hout et al. 2013) using the function "*MDSplot()"*of the "RandomForest" package.

Data from multiple-choice oviposition with important volatiles were analysed with the non-parametric Kruskal –Wallis test followed by the post hoc Dunn's test.

For the results from the field trapping assay, I used a generalised linear model with negative binomial error distribution and log-link to determine whether bait enhanced *S. calcitrans* catches (package 'MASS'(Venables 2002), function 'glm.nb'). Post-hoc Dunnett's tests (R function glht from the package multcomp) were used to compared the number of flies caught by each baited trap with the unbaited control trap (Bretz et al. 2011). I compared the following parameters: (1) the number of flies, (2) the number of fed flies, and (3) the number of gravid females caught by each trap. For the sex ratio parameter (female number/total number of flies), I used ANOVA followed by Dunnett's test. All statistical results were considered significant when $P < 0.05$.

2.4 RESULTS

Gravid female S. calcitrans prefer donkey and sheep dung for oviposition

Gravid female *S. calcitrans* consistently chose donkey and sheep dung as oviposition substrates over the other dung tested. The mean number of eggs laid on each substrate by gravid females was significantly different (Figure 2.1A, Kruskal-Wallis test: H=30.702, d.f = 9, $P < 0.001$). Females laid more eggs on donkey and sheep dung followed by zebra dung than on the dung of cow, camel, buffalo, elephant, or giraffe, grass and wet sand (control). Results obtained in the laboratory with naïve gravid female flies (7-10 days old) mirrored the results obtained in the semi-field assays (Figure 2.1B; H = 59.497, d.f = 9, $P < 0.001$). The higher number of eggs laid on donkey and sheep dung resulted from a higher mean number of batches (Figure 2.1C; H = 54.13, d.f = 9, $P < 0.001$) and mean number of eggs per batch (Figure 2.1D; H= 59.38, d.f = 9, $P < 0.001$).

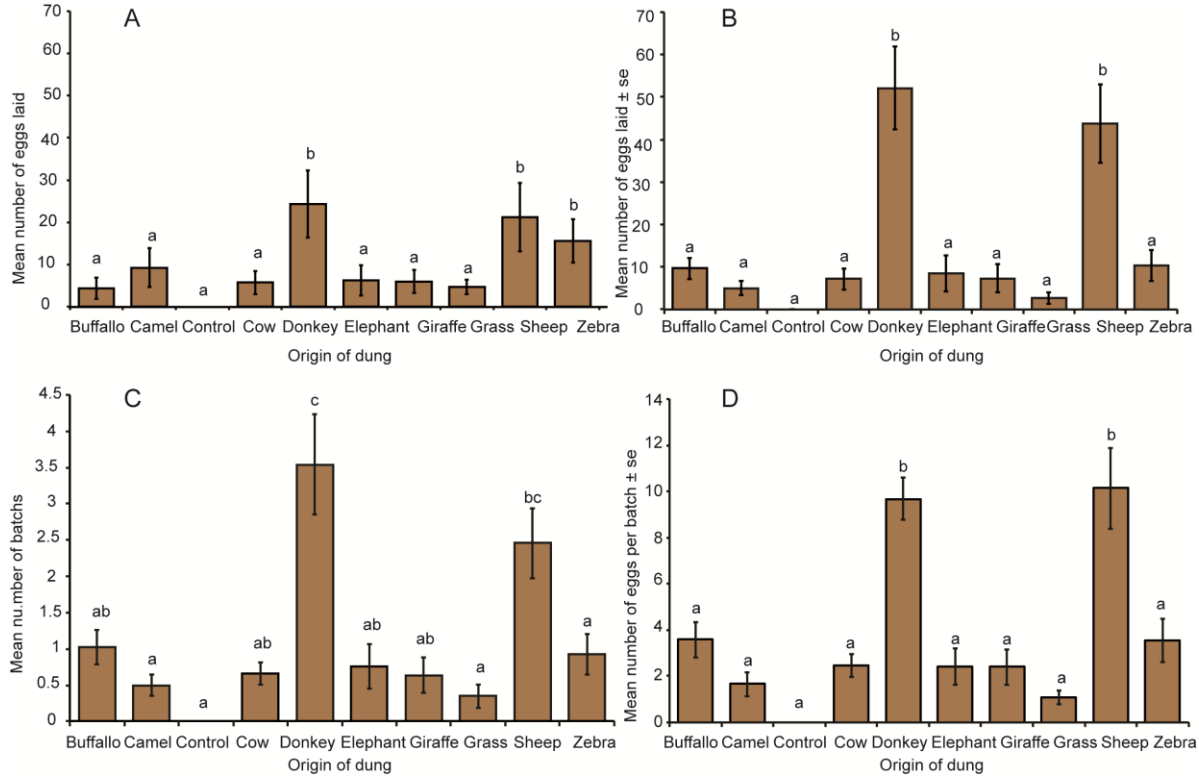

Figure 2.1. Gravid female *S. calcitrans* prefer to oviposit on donkey and sheep dung. **(A)** Mean number of eggs laid on dung of each vertebrate herbivore by wild female S. calcitrans. **(B)** Mean number of eggs laid on dung of each vertebrate herbivore by naïve female S. calcitrans. **(C)** Mean number of egg batches deposited on dung of each vertebrate herbivore by naïve female S. calcitrans. **(D**) Mean number of eggs per batch laid by naïve female S. calcitrans. Error bars indicate standard error of the mean (SEM). Bars with different letters are significantly different from each other (Kruskal-Wallis test followed by Dunn's post hoc test; $P < 0.05$, $n = 10$).

Preferred oviposition substrates enhance fitness of S. calcitrans offspring

Having shown that gravid female *S. calcitrans* prefer to oviposit in the dung of particular vertebrate herbivores, I tested whether female preference led to improved performance by offspring. Egg hatchability, larval and pupal development time, larval weight and pupal weight were recorded as fitness parameters. I found that egg hatchability (GLM, $\chi^2 = 18.355$, d.f = 3, $P < 0.001$), larval development time ($F_{3,36} = 132.2$, $P < 0.001$), larval weight (Day 5: H = 130.52, d.f = 3, $P < 0.001$; Day 10: H = 147.26, d.f = 3, $P < 0.001$), pupal development time or adult emergence ($F_{3,36} = 17.48$, $P < 0.001$), larval growth rate (H = 136.41, d.f = 3, $P < 0.001$), pupal weight ($F_{3,156} = 140.4$, $P < 0.001$) and adult emergence time ($F_{3,36} = 17.48$, $P < 0.001$) were affected by dung type. Egg hatchability was highest in donkey followed by

camel, cow and sheep dung (Figure 2.2A). Development time from egg to pupal stage was significantly shorter in donkey and sheep dung than in cow and camel dung (Figure 2.2B). Larvae that developed in donkey and sheep dung were heavier at days 5 and 10 (Figure 2.2D); consequently, they had the best growth rates (Figure 2.2E). Pupae from donkey and sheep dung weighed more than those from cow and camel dung (Figure 2.2F).

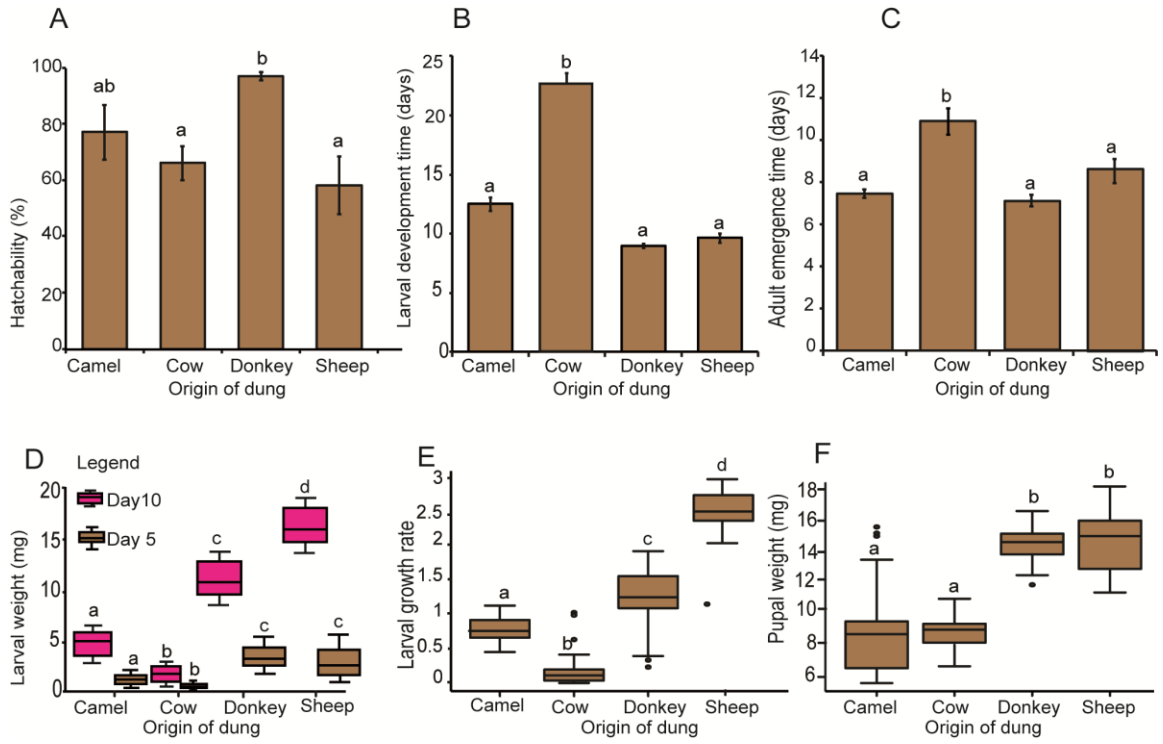

Figure 2.2. Gravid female *S. calcitrans* prefer to oviposit on substrates that enhance the fitness of offspring. (A-C) Bar plots showing: **(A)** egg hatchability, **(B)** larval development time, **(C)** adult emergence times when *S. calcitrans* offspring were reared in camel, cow, donkey, and sheep dung. Error bars represent SEM. Bars with different letters are significantly different from each other (ANOVA followed by SNK post hoc test; $P < 0.05$, n = 10). **(D-F)** Boxplots illustrating: **(D)** larval weight at five (brown) and ten days (pink), **(E)** larval growth rate, **(F)** pupal weight of *S. calcitrans* when raised in camel, cow, donkey, and sheep dung. Boxplot whiskers indicate ± 1.5 interquartile range limits. Box plots with different letters are significantly different from each other [grouped by the Kruskal-Wallis test followed by Dunn's post hoc test for larval weight and larval growth rate, and ANOVA followed by SNK's post hoc test for the pupal weight ($P < 0.05$, n=10)].

Preference-performance relationship positively correlated with the physicochemical composition of preferred substrates

Our results clearly demonstrated that oviposition preference of gravid female *S. calcitrans* contributes to fitness of their offspring. Due to this, I set out to determine whether the preference-performance relationship was correlated with the physicochemical composition of the different oviposition substrates. I analysed the physical properties and micronutrient contents of the two preferred substrates (donkey and sheep dung) and non-preferred substrates (camel and cow dung). There was a significant difference in the physiochemical properties of the different animal dung (Figure 2.3A, MANOVA: Pillai's trace = 2.99, $F_{3,36}$ = 2734, $P < 0.001$). Cow dung had the highest percentage of water content (Figure 2.3A.1) and the lowest percentage of dry matter (Figure 2.3A.2). The pH was lowest in donkey dung (Figure 2.3A.3). I found a lower carbon/nitrogen ratio (Figure 2.3A.4) and a higher amount of copper (Figure 2.3A.9) in sheep dung. Phosphorus (Figure 2.3A.5), potassium (Figure 2.3A.6) and zinc (Figure 2.3A.8), were higher in donkey and sheep dung; while calcium was highest in camel dung (Figure 2.3A.7).

To correlate oviposition choice, fitness and physicochemical parameters, I performed a principal component analysis (PCA). I found these parameters to be highly correlated (Figure 2.3B), indicating that *S. calcitrans* gravid females appear to consider the physiochemical properties of substrates when deciding to oviposit. The first two dimensions of the PCA explained 81.6 % of the total variation. Dimension 1 was associated with the proportion of phosphorus and potassium in dung and accounted for 50 % of the total variation. Dimension 2, which accounted for 31.6 % of the total variation, was highly correlated with carbon/nitrogen ratio and the proportion of copper in dung. To clearly illustrate correlations among measured variables, I constructed a correlogram (Figure 2.3C). Oviposition was positively correlated with larval weight (r = 0.58, $P < 0.001$) and pupal weight (r = 0.64, $P < 0.001$); and negatively correlated with larval developmental time (r = -0.48, $P < 0.001$). Larval weight and pupal weight were positively correlated with the proportion of zinc (r = 0.59, $P < 0.001$; r = 0.75, $P < 0.001$), potassium (r = 0.59, $P < 0.001$; r = 0.73, $P < 0.001$), phosphorus (r = 0.68, $P < 0.001$; r = 0.77, $P < 0.001$), and copper (r = 0.75, $P < 0.001$; r = 0.42, *P =0.0074*) which were more abundant in preferred oviposition substrates. Larval weight and pupal weight were negatively correlated with the proportion of calcium (r = -032, $P = 0.0436$; r = -0.55, $P < 0.001$) and carbon/nitrogen ratio (r = -0.74, $P < 0.001$; r = -0.42, *P = 0.0074*), which were higher in non-preferred oviposition substrates. Larval developmental time was positively correlated with dung water content (r = 0.66, $P <$

0.001) and carbon/nitrogen ratio (r = 0.73, $P < 0.001$), and negatively correlated with the proportion of phosphorus (r = 0.69, $P < 0.001$), potassium (r = 0.66, $P < 0.001$), and copper (r = -0.66, $P < 0.001$), which were again higher in donkey and sheep

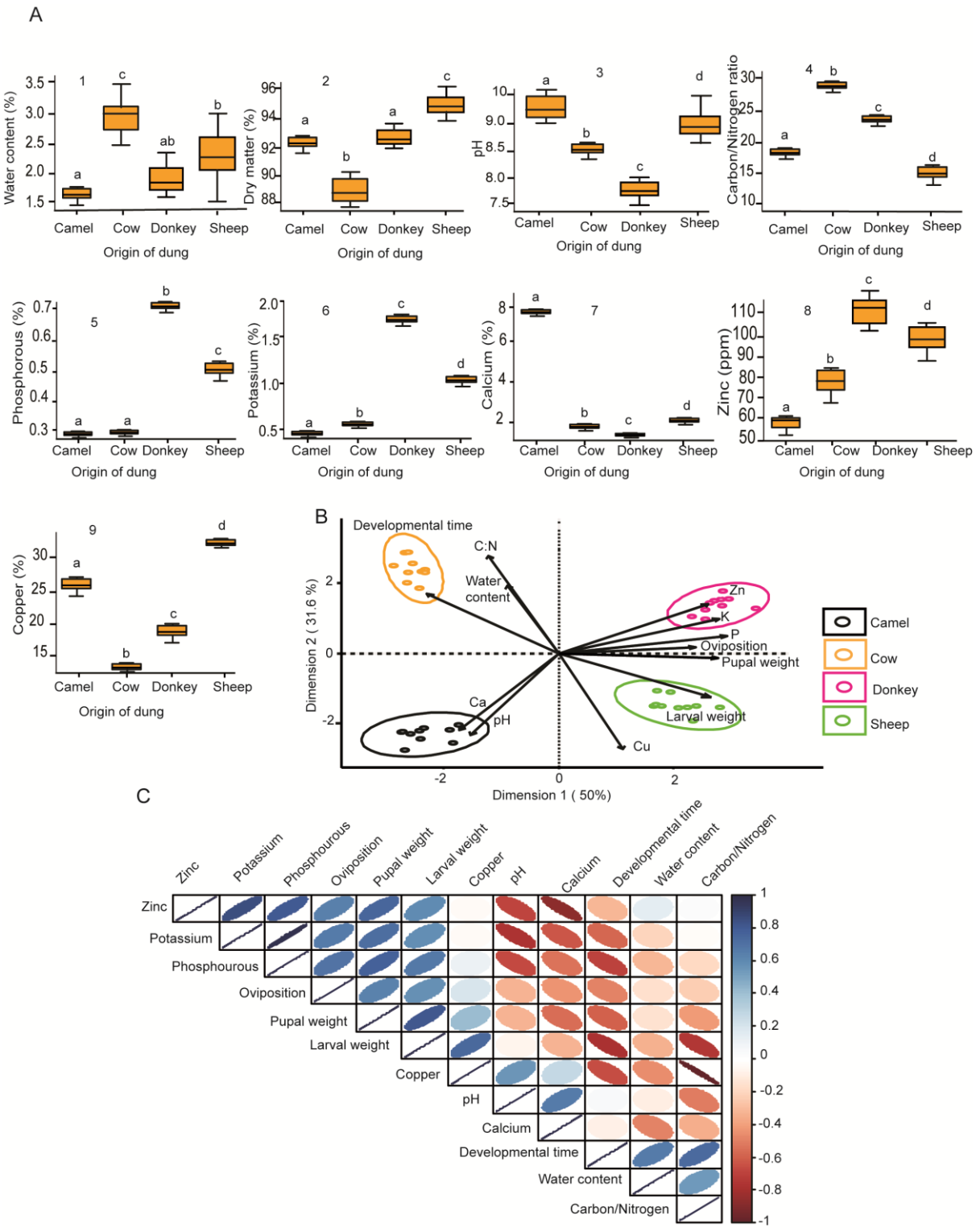

Figure 2.3. Oviposition substrates vary in their physiochemical properties

(A) Boxplot depicting results of physicochemical analysis of camel, cow, donkey and sheep dung: **(1)** water content, **(2)** dry matter, **(3)** pH, **(4)** carbon/nitrogen ratio, **(5)** phosphorus proportion, **(6)** potassium proportion, **(7)** calcium proportion, **(8)** zinc proportion and **(9)** copper proportion. The ends of boxplot whiskers represent the minimum and maximum of all the data. Boxes with different letters are significantly different from each other based on MANOVA followed by SNK post-hoc tests. **(B)** Principal component biplot showing the relation between *S. calcitrans* oviposition preference, larval performance and dung composition. Black ellipse: camel dung; orange ellipse: cow dung; magenta ellipse: donkey dung; and green ellipse: sheep dung. **(C)** Correlogram highlighting the direction and intensity of the correlation between oviposition preference, larval performance traits, and dung composition. Red and blue denote high negative and positive correlation, respectively; white indicates absence of correlation.

Oviposition substrates are distinct in their volatile organic compound (VOC) composition

I determined whether oviposition preference behaviour observed in gravid female *S. calcitrans* (Figure1) was potentially mediated by olfactory cues by using coupled gas chromatography-mass spectrometry (GC-MS) to analyse the VOC composition of all dung types used in the oviposition bioassays. GC-MS analysis identified a wide range of VOCs emitted by the substrates (Figure 2.4A). Using multidimensional scaling ordination, I found that each dung type had a distinct VOC composition (Figure 2.4C).

Signature VOCs of donkey and sheep dung elicit strongest oviposition

I hypothesised that gravid females of *S. calcitrans* use signature VOCs to locate suitable oviposition substrates, and that in our study these signature VOCs are represented by compounds that are abundant and permanently present (most important volatiles) in donkey and sheep dung. To test this hypothesis, I first classified the VOCs of each dung using a classification algorithm method called "random forest" (RF), which has been used successfully in others studies (McCormick et al. 2016; Mansourian et al. 2016; Marneweck et al. 2017b). Based on the function *"importance ()"* embedded in the Random Forest R software package, I obtained the mean decrease in accuracy (MDA) of each volatile present in each dung. As the rule of thumb, VOCs with the highest MDA are, the most important, namely abundant and permanently present across all the replicates of tested vertebrate

herbivore dung. Based on that rule I found that carvone was the most important VOC of buffalo and sheep dung; while *p*-cymene, limonene, β-citronellene, cyperene, *m*-cresol, and camphene were the most important VOCs of camel, cow, donkey, elephant, giraffe, and zebra dung, respectively (Figure 2.4B), with a classification accuracy of 87.9 %.

Having determined the most important VOC of each dung, I next asked if the most important VOC from donkey and sheep dung would stimulate more gravid female *S. calcitrans* to oviposit. To test this, I conducted a multiple-choice oviposition bioassay where gravid females of *S. calcitrans* were presented with wet sand loaded with the synthetic standard of the single most important VOC of each substrate at 10^{-2} v/v dilution. I found that oviposition by *S. calcitrans* varied across the tested VOCs (Figure 2.4D: $H = 25.30$, $df = 6$, $P < 0.001$). In comparison with other media, gravid female *S. calcitrans* laid more eggs on the media loaded with β-citronellene and carvone, which are the most important VOCs of preferred oviposition substrates, donkey and sheep dung, respectively. To verify whether only β-citronellene and carvone led to the observed increase in number of eggs laid, I replaced β-citronellene and carvone with β-caryophyllene and *m*-xylene, which are the most important VOCs from donkey and sheep dung, respectively, and recorded oviposition. Neither β-caryophyllene nor *m*-xylene induced a significant increase in the mean number of eggs laid (Figure 2.4E: $H = 3.78$, $df = 6$, $P = 0.71$). I concluded that β-citronellene and carvone were signature VOCs used by gravid females as olfactory cues to identify the best oviposition

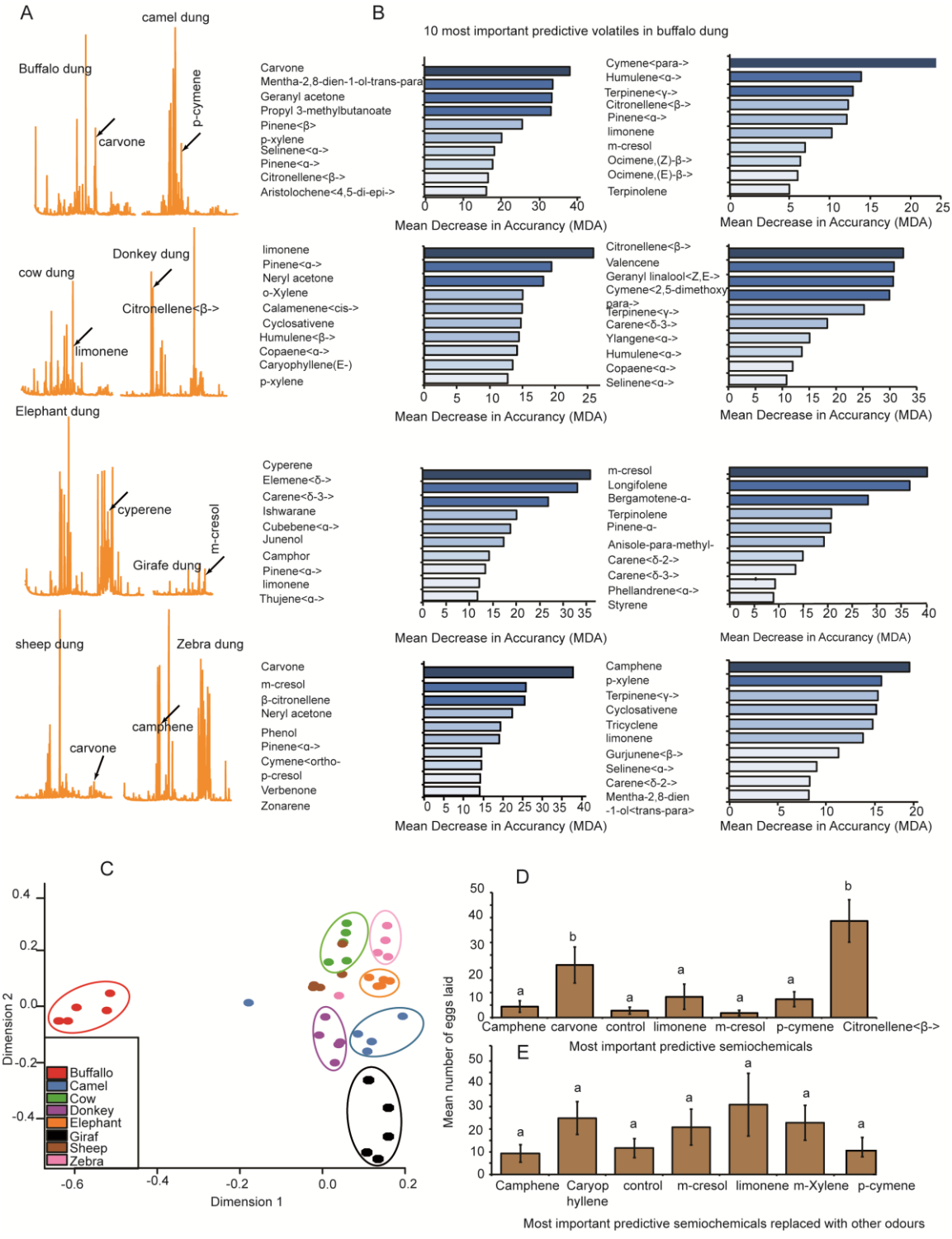
A
camel dung
Buffalo dung
p-cymene
carvone
Donkey dung
cow dung
Citronellene<β->
limonene
Elephant dung
cyperene
m-cresol
Girafe dung
sheep dung
Zebra dung
camphene
carvone
B
10 most important predictive volatiles in buffalo dung
Carvone
Mentha-2,8-dien-1-ol-trans-para
Geranyl acetone
Propyl 3-methylbutanoate
Pinene<β>
p-xylene
Selinene<α->
Pinene<α->
Citronellene<β->
Aristolochene<4,5-di-epi->
Cymene<para->
Humulene<α->
Terpinene<γ->
Citronellene<β->
Pinene<α->
limonene
m-cresol
Ocimene,(Z)-β->
Ocimene,(E)-β->
Terpinolene
Mean Decrease in Accurancy (MDA)
limonene
Pinene<α->
Neryl acetone
o-Xylene
Calamenene<cis->
Cyclosativene
Humulene<β->
Copaene<α->
Caryophyllene(E-)
p-xylene
Citronellene<β->
Valencene
Geranyl linalool<Z,E->
Cymene<2,5-dimethoxy
para->
Terpinene<γ->
Carene<δ-3->
Ylangene<α->
Humulene<α->
Copaene<α->
Selinene<α->
Cyperene
Elemene<δ->
Carene<δ-3->
Ishwarane
Cubebene<α->
Junenol
Camphor
Pinene<α->
limonene
Thujene<α->
m-cresol
Longifolene
Bergamotene-α-
Terpinolene
Pinene-α-
Anisole-para-methyl-
Carene<δ-2->
Carene<δ-3->
Phellandrene<α->
Styrene
Carvone
m-cresol
β-citronellene
Neryl acetone
Phenol
Pinene<α->
Cymene<ortho-
p-cresol
Verbenone
Zonarene
Camphene
p-xylene
Terpinene<γ->
Cyclosativene
Tricyclene
limonene
Gurjunene<β->
Selinene<α->
Carene<δ-2->
Mentha-2,8-dien
-1-ol<trans-para>
C
Dimension 1
Dimension 2
Buffallo
Camel
Cow
Donkey
Elephant
Giraf
Sheep
Zebra
D
Mean number of eggs laid
Camphene
carvone
control
limonene
m-cresol
p-cymene
Citronellene<β->
Most important predictive semiochemicals
E
Camphene
Caryop
hyllene
control
m-cresol
limonene
m-Xylene
p-cymene
Most important predictive semiochemicals replaced with other odours

Figure 2.4. **The most important volatiles from donkey (β-citronellene) and sheep (carvone) dung elicit the strongest oviposition response by *S. calcitrans* gravid females.** **(A)** Representative GC–MS chromatogram of each vertebrate herbivore dung and most important semiochemicals. **(B)** Multidimensional scaling (MDS) plot showing the segregation of vertebrate herbivore dung based on their VOC composition. **(C)** Histogram showing the classification of the ten most important VOCs from vertebrate herbivore dung based on the Mean Decrease in Accuracy (MDA) of the Random Forest analysis. VOC associated with the darkest histogram has the highest MDA value and consequently, the most important. **(D-E)** Bar plots representing: **(D)** mean number of eggs laid by gravid female *S. calcitrans* on each oviposition medium (wet sand) loaded with the most important VOC of each vertebrate herbivore dung. Error bars represent SEM. **(E)** mean number of eggs laid by gravid female *S. calcitrans* on each oviposition medium (wet sand) loaded with the most important chemical volatile of each vertebrate herbivore dung with the replacement of β-citronellene and carvone by β-caryophyllene and m-xylene, respectively. Bars with different letters are significantly different from each other (Kruskal-Wallis test followed by Dunn's post hoc test; $P < 0.05$). Error bars represent SEM.

β- Citronellene and carvone enhance trap catch of S. calcitrans

Having identified the VOCs that stimulate gravid female *S. calcitrans* to oviposit more on donkey and sheep dung, I assessed their attractiveness under field conditions. A Latin square design experiment was performed at Mpala Research Centre (www.mpala.org) located in Laikipia County, Kenya (Figure 2.5A) using monoconical traps (Figure 2.5B). Traps were baited with different VOCs (Figure 2.5C) and rotated daily to account for any bias resulting from trap location (Perry et al. 1980). Each treatment consisted of an undiluted solution of β-citronellene, carvone, Blend A (carvone +β-citronellene), Blend B (β-citronellene + valencene), Blend C (carvone + valencene + γ- terpinene), *m*-cresol (positive control: already known to attract *S. calcitrans*; see Tangtrakulwanich et al (2015), or a negative control (unbaited trap).

Overall, I caught more *S. calcitrans* [8702 (85%)] than other insects [1739 (15%)] (χ^2 = 4643.6, df = 1, p < 0.001). Other insects mainly comprised house flies, *Musca domestica* [1706 (98.1%)]. The number of *S. calcitrans* caught significantly varied with VOC treatment (GLM.nb: LR = 15.74, df = 6, $P < 0.05$). The mean number of *S. calcitrans* caught by traps

baited with β-citronellene, Blend A, Blend B, carvone, Blend C and *m*-cresol were respectively 3.9, 3.3, 3.30, 2.6, 2.5 and 2.4 times more than those caught in unbaited traps (Figure 2.5 D).

Irrespective of VOC treatment, I caught significantly more blood fed (58.6%)) than unfed *S. calcitrans* (41.41%) ($\chi^2 = 256.5$, df = 1, $P < 0.001$). The number of blood-fed flies varied significantly among VOC treatments (GLM.nb: LR = 9.83, df = 6, P < 0.05). Traps baited with β-citronellene, Blend A, Blend B, carvone and Blend C caught significantly more blood-fed *S. calcitrans* than the unbaited trap. Conversely, trap catches of blood-fed *S. calcitrans* with *m*-cresol-baited and -unbaited traps were not significantly different (Figure 2.5 E).

During the entire period of trapping, I caught significantly more female *S. calcitrans* (65%) than males (35%) ($\chi^2 = 741.39$, df = 1, $P < 0.001$). The VOC treatment significantly affected the sex ratio (female to total number of individuals ratio) of caught flies ($F_{6,42}= 3.74$, $P = 0.0045$). The sex-ratio was female-biased in traps baited with β-citronellene, carvone, Blend B, Blend A, and *m*-cresol, while the unbaited traps caught the same number of females and males (Figure 2.5F).

The number of gravid females caught significantly differed among the VOC treatments (GLM.nb: LR= 14.35, df = 6, $P < 0.05$). Among all the VOC treatments, only traps baited with β-citronellene caught significantly more gravid females of *S. calcitrans* than the unbaited traps. The mean number of gravid flies caught by traps baited with carvone, *m*-cresol, Blend A, Blend B, Blend C was not significantly different from the unbaited trap catch (Figure 2.5G).

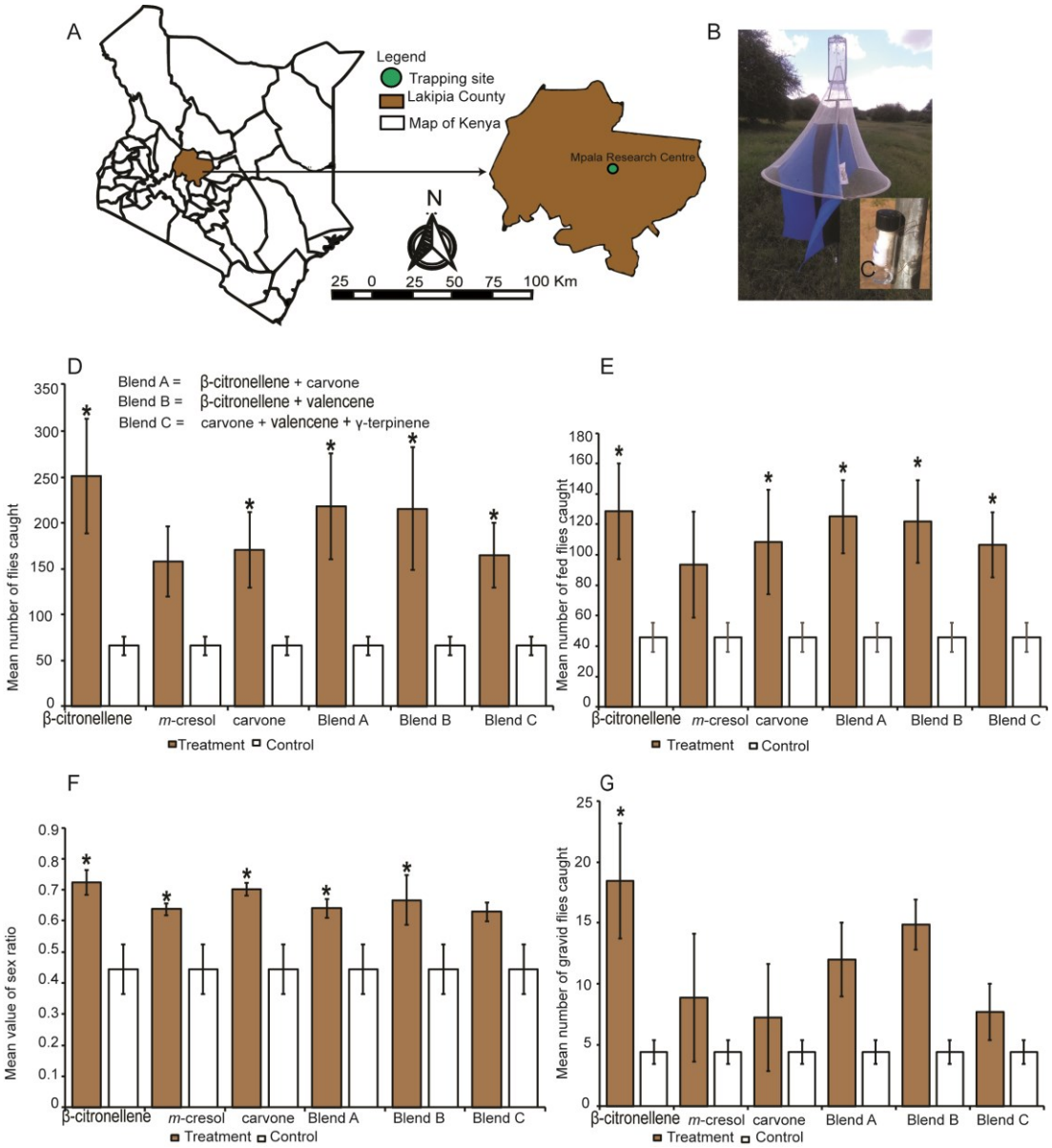

Figure 2.5. β-citronellene and carvone significantly enhance *S. calcitrans* trap catch. (**A**)Trapping site map, (**B**) Monoconical trap (C) Cotton roll dispenser in 4ml vial with perf orated cap. (**D-G**) bar plots depicting: (**D**) the mean number of *S.calcitrans* caught, (**E**) the mean number of *S.calcitrans* blood fed flies caught, (**F**) the mean value of *S.calcitrans* sex r atio, (**G**) the mean number of *S.calcitrans* gravid females caught. Error bars represent SEM ., treatment with an asterisk (*) above the error bar are significantly different from the nega tive control (unbaited trap) [Dunnett's t-test ($P < 0.05$, n=7)].

2.5 DISCUSSION

Our results demonstrate that gravid female *S. calcitrans* exhibit a preference-performance oviposition behaviour that is mediated by olfactory cues. Additionally, these results demonstrate that oviposition preference is associated with better fitness of *S. calcitrans* immature stages.

Results from the oviposition preference bioassay revealed that gravid female *S. calcitrans* preferred to lay eggs on donkey dung followed by sheep dung. This has also been reported by Hafez & Gamal-Eddin (1959) but the causes for this preference and the fitness benefits were not investigated. I demonstrated that oviposition preference by female *S. calcitrans* was related to better fitness of *S. calcitrans* immature stages. For instance, larvae and pupae of *S. calcitrans* were heavier and developed more rapidly on donkey and sheep dung. This may reflect the higher nutritional value of these specific substrates for *S. calcitrans* (Frago and Bauce 2014). I show that nitrogen, zinc, potassium, phosphorous and copper content were significantly higher in donkey and sheep dung. These elements were positively correlated with larval and pupal weights and negatively correlated with developmental time. The positive effect of these elements in insect fitness is widely acknowledged in the literature (Schofield et al. 2002; Willott and Tran 2002; Bhattacharya and Kaliwal 2005; Rizvi and Raman 2017). Similarly, Perkins et al (2004) demonstrated that augmentation of the diet with phosphorus increased the growth rate of the tobacco hornworm, *Manduca sexta* (L) (Lepidoptera: Sphingidae).

Evidence for the preference-performance relationship has been demonstrated in other insect groups although studies have been heavily biased towards herbivorous insects. For example, Heisswolf et al. (2005) found that females of the monophagous beetle *Cassida canaliculata* (Coleoptera: Chrysomelidae) preferred to oviposit on larger host plants (rich in nitrogen), which then led to improved larval performance and survival in comparison with larvae developing on smaller host plants. Similarly, Chen et al. (2008) have shown that cotton with a higher level of nitrogen is suitable for oviposition and development of larvae of the beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae). When offered a choice between two cultivars of *Brassica oleracea* (Derby Day and Drago), female diamondback moths, *Plutella xylostella* (Lepidoptera: Plutellidae) laid more eggs on the cultivar with

lower glucosinolate concentration, which also maximised larval performance (Staley et al. 2009).

Our results revealed that the preference-performance behaviour observed in *S. calcitrans* was driven by olfactory cues. I demonstrated that although vertebrate herbivore dung contained a plethora of VOCs [more than 45 each (Fig. 2.4A)], *S. calcitrans* used a single semiochemical to select an oviposition site. For instance, only one VOC identified using random forest analysis was enough to mimic the presence of preferred or unpreferred vertebrate herbivore dung. As predicted, the most important VOC from donkey dung (β-citronellene) was enough to mimic the stimulatory effect of the substrate. On the other hand, media loaded with carvone did not significantly differ from the other media (Figure 5D). This result shows that to mimic the response of gravid *S. calcitrans* to sheep dung, some components might be missing. Several studies report that a single compound might be enough for females to detect oviposition substrates and initiate oviposition(Dweck et al. 2013; Pagadala Damodaram et al. 2014), while in other cases a signature blend of VOCs might be required for the same outcome(Kamala Jayanthi et al. 2014; Riffell et al. 2014). In the absence of β-citronellene and carvone, *S. calcitrans* gravid females failed to exhibit a preference for any substrate. Instead, they laid eggs randomly on each presented medium including those that led to poor fitness, demonstrating the importance of β-citronellene and carvone in oviposition-site selection. However, it is not clear how these semiochemicals relate to the nutrient value of the substrates. It is likely that other sensory modalities play a role, such as taste. Jeanbourquin & Guerin(Jeanbourquin and Guerin 2007) reported the presence of β-citronellene in horse dung (like donkey, in the family Equidae) with electrophysiological activity in *S. calcitrans* antennae, but the authors did not go on to explore the effect of this chemical on *S. calcitrans* behaviour.

Synthetic semiochemicals eliciting a specific behaviour in insects can enhance insect catches when used as a bait in field trapping systems(Norin 2007; Logan and Birkett 2007; Reisenman et al. 2016). This was confirmed in our field work when I baited monoconical traps with β-citronellene, carvone, Blend A, Blend B, and Blend C. In this experiment, *S. calcitrans* represented 85% of insects caught, which supports the results of Mihok et al. (1995) and Tunnakundacha et al. (2017) who reported that monoconical traps are efficient for trapping flies in the subfamily Stomoxyinae. Traps baited with β-citronellene, carvone, Blend A, Blend B, and Blend C captured more flies than unbaited traps (negative control) and traps baited with *m*-cresol (positive control) (Figure 2.5D). When checked for feeding

status, traps baited with the same VOC treatments captured more engorged flies than the unbaited traps (Figure 2.5E). A possible explanation for this result is that vertebrate herbivore dung is more likely to be visited by fed females for oviposition. Additionally, males of *S. calcitrans* are also attarcted to dung-derived semiochemicals, most probably to look for females for mating. Interestingly, when I checked for gravid status, I found that only traps baited with β-citronellene caught significantly (4.5 times) more gravid female *S. calcitrans* than unbaited traps. Also, traps baited with blends containing β-citronellene [Blend A (carvone+β-citronellene) and Blend B (β-citronellene+valencene)] captured more gravid flies than traps baited with carvone, *m*-cresol, and Blend C (carvone + valencene + γ-terpinene) (Figure 2.5G). This clearly shows that, either as a single or combined with other dung volatiles, β-citronellene efficiently attracts gravid female *S. calcitrans.*

2.6 CONCLUSION

In conclusion, gravid female *S. calcitrans* exhibit a preference-performance oviposition behaviour driven by signature odours emanating from vertebrate herbivore dung. Larvae and pupae developing from eggs laid on preferred substrates exhibit higher fitness than those that develop on non-preferred substrates. Furthermore, a single or blend of signature VOCs such as β-citronellene and carvone increased *S. calcitrans* female attraction and stimulated oviposition both under laboratory and field conditions. The high level of female-biased attraction, including high levels of gravid female and blood-fed flies, to the semiochemical β–citronellene associated with oviposition substrates, is promising for its potential use in the management of *S.calcitrans*.

CHAPTER 3 Stable flies, Stomoxys calcitrans L. (Diptera: Muscidae), improve offspring fitness by avoiding oviposition substrates with competitors or parasites

This chapter has been submitted for peer review as:

Baleba, S. B. S., Torto, B., Masiga, D., Getahun, M. N. and Weldon, C. W. Stable flies, *Stomoxys calcitrans* L. (Diptera: Muscidae), improve offspring fitness by avoiding oviposition substrates with competitors or parasites. Submitted to: *BMC Ecology*.

3.1 ABSTRACT

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, I assessed whether the presence of conspecific, heterospecific larvae and parasites influenced oviposition decisions by the stable fly, *Stomoxys calcitrans* (Linneaus). Using dual and multiple-choice oviposition bioassays, I found that gravid female *S. calcitrans* avoided substrates with conspecific larvae, the larvae of house flies, *Musca domestica* (Linneaus), and the mite *Macrocheles muscaedomesticae* (Scopoli). Avoidance of conspecific and heterospecific larvae persisted in the dark, suggesting that this behaviour is mediated by olfactory cues. When I reared *S. calcitrans* in the presence of conspecific larvae and the larvae of house flies at different densities I found that this negatively affected emergence time, larval weight, larval survival, pupal weight, pupal survival, and adult weight. I 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. I recommend that the olfactory 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.

3.2 INTRODUCTION

Oviposition site selection is a complex task in the life history of all holometabolous insects. As the egg stage is immobile and most adult insects do not practice maternal care, gravid females need to lay eggs on a substrate that maximises the fitness of their offspring. 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 and Roitberg 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* (Linneaus) 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* (Linneaus), 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 behaviour towards each other (wing waving, fast running towards 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 behaviour 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* (Linneaus), 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 besnoit*), 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. (2010) estimated that *S. calcitrans* lead to economic losses of around $2.2 billion per year.

Gravid females of *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 (Linz et al. 2013; Conchou et al. 2017). Decaying plant-based material is also an important ecological niche for other invertebrate species (Sladecek et al. 2017) such as the house fly, *Musca domestica* (Linneaus) (Broce and Haas 1999) and the mite *Macrocheles muscaedomesticae* (Scopoli) (Jalil et al. 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 larvae stages of flies (Axtell 1963). 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 (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, I determined how competition and parasitism affected oviposition decisions by gravid female *S. calcitrans*. I hypothesised they would avoid substrates with conspecific heterospecific larvae and parasites to protect their offspring from intra- or inter-specific competition and parasitism. To test this hypothesis, I 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, I 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.

3.3 MATERIAL AND METHODS

Study animals and substrates

Stomoxys calcitrans flies were obtained from a single culture established for approximately one 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; ≈ 1600 m above sea level). 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 were collected from the *icipe* rabbit breeding unit. After exposure for 24 hrs I transferred these containers to another cage (75×60×45 cm), and I monitored the development of the larval and pupal stages until adult emergence. Emerged flies were fed twice per day (0800 and 1600 hours) 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* (Fig. 5Ai) were harvested directly from unsterilized rabbit dung collected at the *icipe* rabbit breeding unit. I 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^{o}C and 65±5% relative humidity. The photoperiod was 12L:12D unless otherwise stated.

Oviposition in the presence of conspecific and heterospecific larvae

I performed multiple-choice oviposition bioassays in cages (75×60×45 cm). In each cage, I allowed 20 gravid female *S. calcitrans* to oviposit on four types of substrate presented simultaneously: (1) rabbit dung, (2) rabbit dung with ten *S. calcitrans* third instar larvae, (3) rabbit dung with ten *M. domestica* third instar larvae, and (4) rabbit dung with ten *S. calcitrans* and ten *M. domestica* third instar larvae. For each replicate, I counted the number of eggs laid on each treatment 24 hrs after setting-up the bioassay. During all experiments performed in the laboratory, I 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 ten times.

Oviposition in response to abundance of conspecific and heterospecific larvae

In the previous bioassay, I found that gravid female *S. calcitrans* avoided laying eggs on a substrate containing conspecific larvae (Fig. 3.1A). Here, I aimed to establish whether avoidance of conspecifics was dependent on the number of individuals present on a substrate. I performed a density-dependent response oviposition bioassay where I 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. I found that female *S. calcitrans* avoided all the substrates with conspecific larvae (Fig. 3.1Bi). Afterwards, I assessed whether visual or chemical cues mediated conspecific avoidance. To do so, I followed the protocol developed by Yang and Shiao (2012) in two blow flies, *Chrysomya megacephala* (Fabricus) 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 ten gravid female *S. calcitrans*, I introduced two treatments: (1) rabbit dung and (2) rabbit dung+40 third instar *S. calcitrans* larvae. I found that gravid female *S. calcitrans* significantly avoided the substrate containing conspecific larvae (Fig.

3.1Bii). I also assessed whether gravid female *S. calcitrans* could avoid a substrate already used by conspecific larvae. In a two-choice bioassay, I 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 (one-week fermented rabbit dung). Each bioassay described above was performed for 24 hrs after which I counted the number of eggs laid on each substrate treatment. Each bioassay was replicated ten times.

The same series of experiments was then performed using *M. domestica* larvae. In the multiple-choice oviposition bioassay, I found that gravid females of *S. calcitrans* laid the same number of eggs laid on the control substrate and the substrate with larvae of *M. domestica* did not differ significantly (Fig. 3.1 A). I therefore sought to elucidate whether this behaviour 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*.

Oviposition in the presence of parasites

To evaluate how oviposition by *S. calcitrans* is affected by the presence of parasites in dung, I 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 *Macrocheles muscaedomesticae*. I counted the number of eggs laid on each substrate after 24 hrs and I replicated the experiment 10 times. I found that *S. calcitrans* gravid females laid fewer eggs on the substrate with mites (Fig. 3.2A). To find out if this behaviour was driven by visual or olfactory cues, I repeated the two-choice bioassays in darkness as described previously. Ten replicates of these bioassays were performed:

Costs 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, I hypothesised 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*, I reared first instar larvae in groups of 5, 15 and 25 in plastic cups filled with 50 g of rabbit dung. Daily, I 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. I replicated each treatment ten times. For the larval weight parameter, I individually weighed all the larvae present on each density treatment across times.

To test the fitness consequences for *S. calcitrans* developing in the presence of interspecific competition, I 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 ten times. Here, I 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, I 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 colour.

Cost of developing in a substrate with parasites

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

Data analysis

All analyses were performed using the R environment for statisitcal 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, I 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, I used the paired Mann-Wilcoxon test. Number of eggs laid by *S. calcitrans* in the presence or absence of the mite *M. muscaedomesticae* were normally distributed, whether tested in the light or dark. To analyse these data, I 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 I 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 (I 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, I 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' (Matthew and Lau 2013). I 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), I used generalised linear models (GLM) with binomial distribution to test whether these variables were affected by larval density (Warton and Hui 2011). I established the significance of the model using analysis of deviance (chi-squared) tests. Using the "emmeans" R package (Lenth, 2018), I 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 I 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 I ran ANOVA tests followed by DTK post hoc tests. I used a generalised 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, I used a generalised linear model (GLM) with binomial distribution and an analysis of deviance (chi-squared) test. To compare survivorship of adult flies with mites and without mites, I performed Kaplan–Meier survival analysis with the Mantel-Cox log-rank chi-squared test using the R package "survival" (Therneau 2015).

3.4 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 (Fig. 3.1A; H=11.45, df = 3, *P= 0.009*). This avoidance behaviour was not noted when the substrate was already colonised 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 (Fig. 3.1Bi, H=14.13, df = 4, *P= 0.007*). Under dark conditions, females completely avoided the substrate with conspecific larvae (Fig. 3.1Bii: U=0, *P<0.001*). They also preferred the unused substrate over the substrate used by conspecific larvae (Fig. 3.1Biii: U=55, *P= 0.002*).

An incremental increase in the number of heterospecific larvae colonising 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 (Fig 3.1 Ci: 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 (Fig 3.1Cii: U=55, *P= 0.002*). Similarly, significantly fewer

eggs were laid on the substrate used for *M. domestica* development than the control (Fig. 3.1Ciii: U=42, *P= 0.019*).

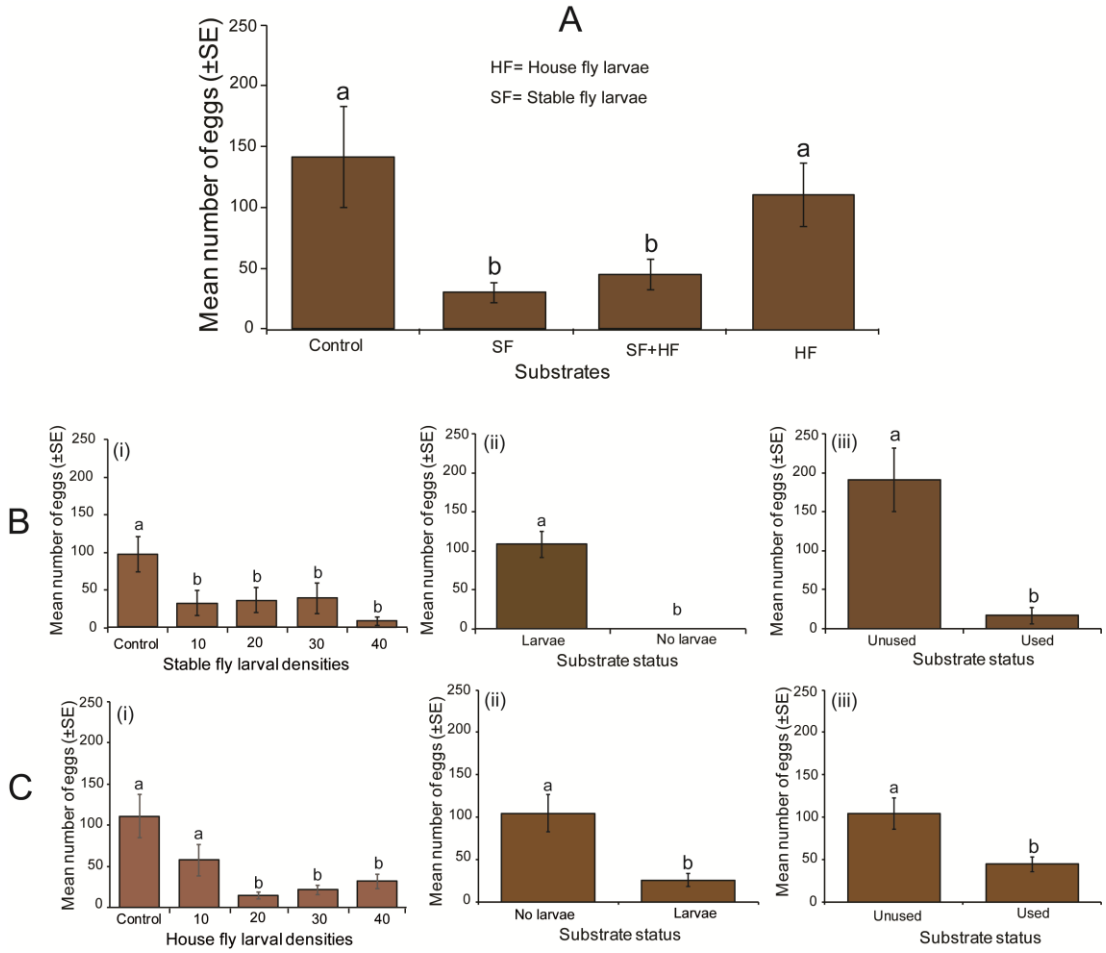

Figure 3.1. Influence of the presence of *S. calcitrans* and *M. domestica* larvae on oviposition decisions by *S. calcitrans*. **(A)** Bar chart showing the mean number of eggs laid by gravid female *S. calcitrans* in the presence of conspecific larvae and the larvae of the house fly (HF), *M. domestica*. **(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, (ii) with unused and used substrates. **(C)** Bar charts depicting the mean number of eggs laid by gravid female *S. calcitrans* in the presence of *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).

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 (Fig. 3.2A: 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 (Fig. 3.2B: t= - 0.14, df=9, *P= 0.82*).

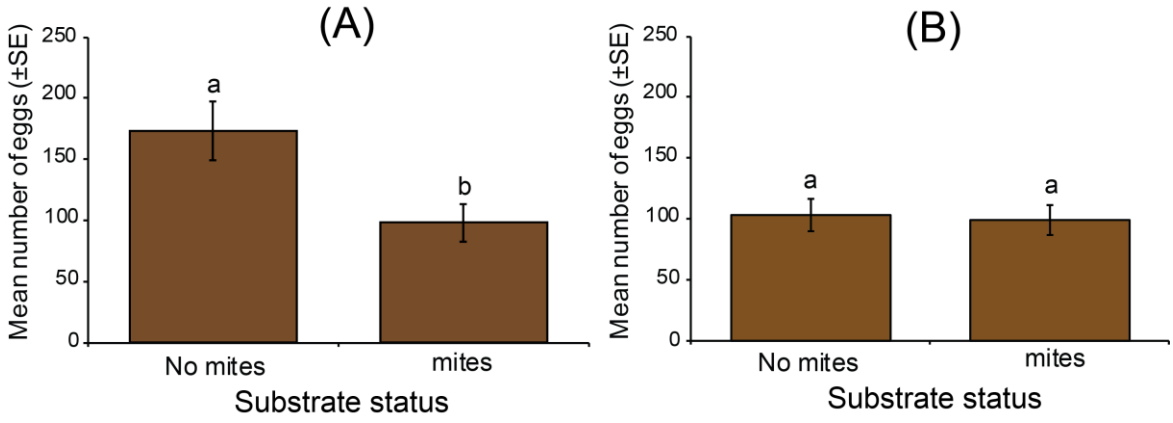

Figure 3.2. Influence of the presence of the mite *Macrocheles muscaedomesticae* on oviposition decisions by *S. calcitrans*. (A) Bar chart depicting the mean number of eggs laid by *S. calcitrans* on substrates with and without mites under light (Paired t-test, P<0.05, n=10). (A) Bar chart depicting the mean number of eggs laid by *S. calcitrans* on substrates with and without mites in darkness (Paired t-test, *P>0.05*, n=10). Error bars indicate the standard error of the mean. Treatments with different lowercase letters are significantly different from each other.

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 (Fig. 3.3A; H=7.73, df=2, *P= 0.02*). Also, larval density significantly influenced larval weight (Day 4: $F_{2\text{-}63}$= 3.5, *P= 0.035*; Day 7: H= 15.60, df= 2, *P< 0.001*; Day 10: $F_{2\text{-}50}$= 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 (Fig. 3B). Despite differences in the body weight of larvae, the percentage of larvae reaching the pupal stage did not differ statistically (Fig. 3.3C: GLM, χ^2 = 2.27, d.f = 2*, P=0.32*). Pupal weight differed significantly across the larval density treatments (Fig. 3.3D, 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 (Fig. 3.3E; GLM, $\chi^2 = 17.06$, d.f = 2, *P<0.001*). Larval density also significantly affected adult weight ($F_{2\text{-}75} = 4.50$, *P<0.05*). Adults from larvae reared in a group of 5 and 15 weighed more than those reared in a group of 25 (Fig. 3.3F).

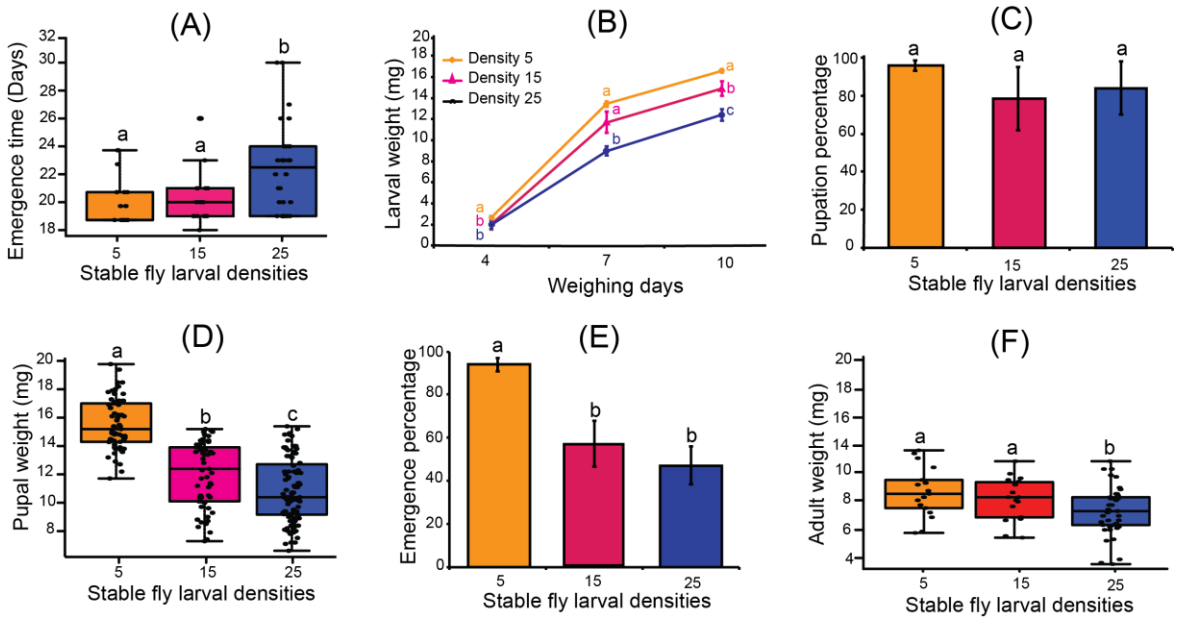

Figure 3.3. Effect of conspecific larvae density on fitness traits of *S. calcitrans*. (A) Boxplot showing *S. calcitrans* emergence time across the different larval densities (Kruskal-Wallis test followed by Dunn's post-hoc tests, $P<0.05$, n=10). **(B)** Line graph illustrating the change of *S. calcitrans* larvae weight across the different larval densities (ANOVA test followed by SNK's post hoc test, $P<0.05$, n=10), 7 (Kruskal-Wallis test followed by Dunn's post hoc test, $P<0.05$, n=10) and 10 (ANOVA test followed by SNK's post hoc test, $P<0.05$, n=10). **(C)** Bar charts depicting the mean percentage of *S. calcitrans* larvae reaching the pupal stage across the different larval densities (GLM with binomial distribution followed by Tukey post-hoc mean separation test, $P<0.05$, n=10). **(D)** Boxplot showing *S. calcitrans* pupal weight across the different larval densities (Kruskal-Wallis test followed by Dunn's post hoc test, $P<0.05$, n=10). **(E)** Bar chart illustrating the mean percentage of *S. calcitrans* emerging as adults from the pupal stage across the different larval densities (GLM with binomial distribution followed by Tukey post hoc mean separation test, $P<0.05$, n=10). **(F)** Boxplot depicting *S. calcitrans* adult weight across the different larval densities (ANOVA test followed by DTK's post hoc test, $P<0.05$, n=10). On each boxplot, the minimum and maximum values of all the data are represented by the ends of boxplot whiskers. On the line graph and bar charts, error bars indicate the standard error of the mean. Treatments with different lowercase letters are significantly different from each other.

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 ($F_{4\text{-}104}$= 4.45, *P= 0.002*), pupation percentage (GLM, χ^2 = 44.51, d.f = 4, *P<0.001*), pupal weight (H= 35.54, df= 4, *P< 0.001*), emergence percentage (GLM, χ^2 = 24.38, d.f = 4, *P<0.001*) and adult weight ($F_{4\text{-}57}$= 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 (Fig. 3.4A). When *S. calcitrans* were reared with 30 or 40 *M. domestica* larvae their larval weight was less than in other treatments (Fig. 3.4B). The percentage of *S. calcitrans* larvae that reached the pupal stage was higher on the substrate devoid of house fly larvae (Fig. 3.4C). Individuals from the substrate without larvae or with ten *M. domestica* larvae had greater pupal weight (Fig. 3.4D) and emergence percentage (Fig. 3.4E) than those from other treatments. Adults from the substrate without house fly larvae were heavier than those reared from other treatments (Fig 3.4F).

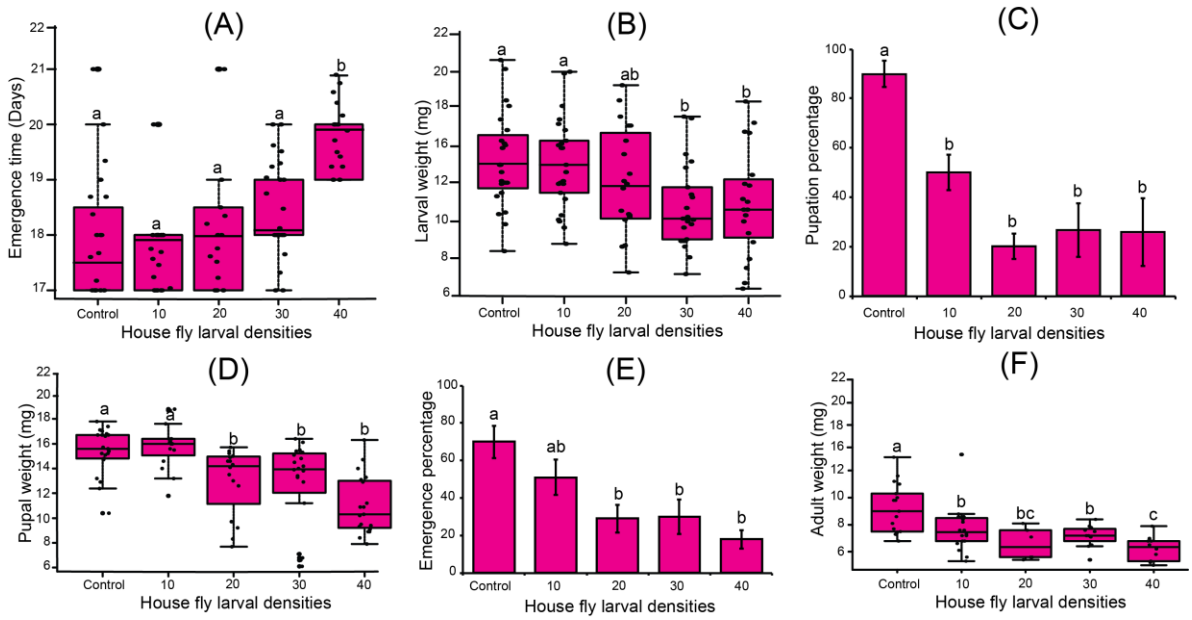

Figure 3.4. Effect of *M. domestica* larval density on fitness parameters of *S. calcitrans*. **(A)** Boxplot showing the variation of *S. calcitrans* emergence time across the different larval densities (Kruskal-Wallis test followed by Dunn's post hoc test, $P<0.05$, n=10). **(B)** Boxplot illustrating the change of *S. calcitrans* larvae weight across the different larval densities (ANOVA test followed by DTK's post hoc test, $P<0.05$, n=10). **(C)** Histograms depicting the pupation percentages of *S. calcitrans* across the different larval densities (GLM with binomial distribution followed by Tukey post hoc mean separation test, $P<0.05$, n=10), **(D)** Bar chart showing the variation of *S. calcitrans* pupae weight across the different larval densities (Kruskal-Wallis test followed by Dunn's post hoc test, $P<0.05$, n=10). **(E)** Bar chart illustrating the variation of *S. calcitrans* emergence percentage across the different larval densities (GLM with binomial distribution followed by Tukey post hoc mean separation test, $P<0.05$, n=10). (**F)** Boxplot depicting the variation of *S. calcitrans* adult weight across the different larval densities (ANOVA test followed by DTK's post hoc test, $P<0.05$, n=10). On each boxplot, the minimum and maximum values of all the data are represented by the ends of boxplot whiskers. On the bar charts, the error bars indicate the standard error of the mean (SEM). Treatments with different lowercase letters are significantly different from each other

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 (Fig 3.5B: GLM, χ^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 (Fig. 3.5C: GLM, χ^2= 171.03, df=1, *P<0.001*). Mite infestation of the substrate negatively affected adult survival (Fig. 3.5D; log-rank test, χ^2= 6.4, *P=0.011*).

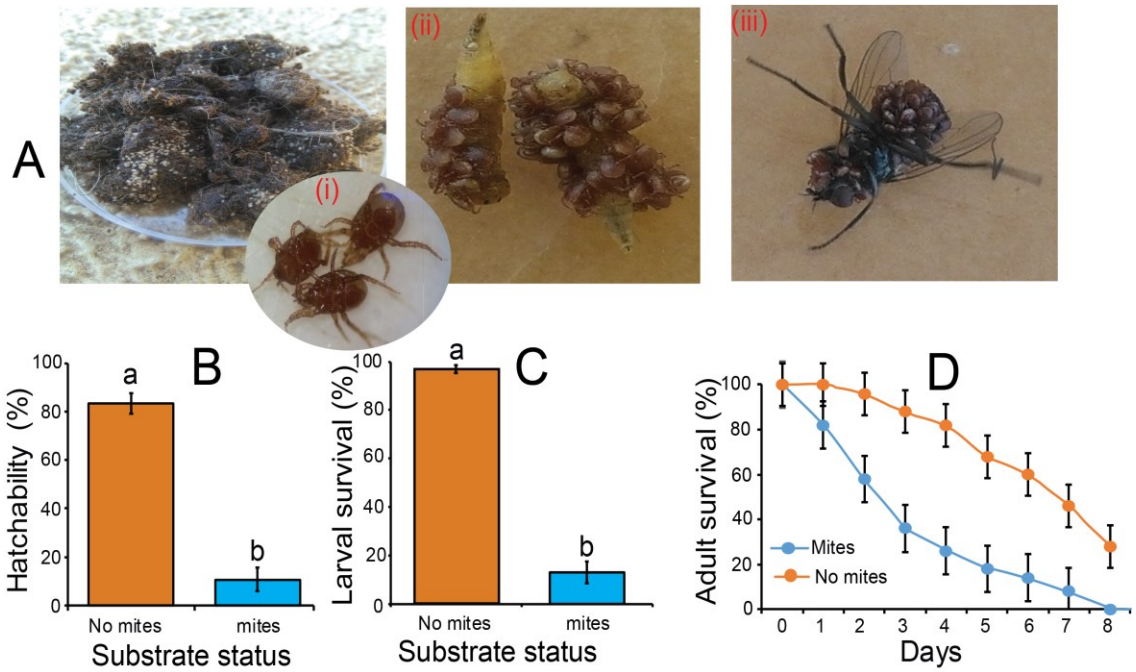

Figure 3.5. Effect of the mite *M. muscaedomesticae* on *S. calcitrans* survival. (A) Adult *M. muscaedomesticae* (i) aggregated on dead *S. calcitrans* larvae as well as (ii) dead and (iii) live adult *S. calcitrans*. **(B-C)** Bar charts showing hatchability of *S. calcitrans* eggs (B) and larval survival (C) on substrates with and without mites (GLM with binomial distribution followed by the analysis of deviance test, *P<0.05*, n= 10). **(D)** Kaplan-Meier curve showing survivorship over time in adult of *S. calcitrans* with or without mite infestation (Mantel-Cox log-rank χ^2 test, *P<0.05*, n=5). Error bars indicate the standard error of the mean. Treatments with different lowercase letters are significantly different from each other.

3.5 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. I demonstrated that ten conspecific larvae were enough to trigger this avoidance, whereas in the presence of *M. domestica*, avoidance began when there were more than ten 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 olfactory 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 *C. megacephala*, when liver with and without larvae of *C. rufifacies* were provided to gravid females, they oviposited on liver without larvae (Yang and Shiao 2012). I suggest that *S. calcitrans* and *M. domestica* larvae may produce one or several chemical cues that strongly deter the oviposition behaviour of *S. calcitrans* gravid females. 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 (Chang 2013), deter oviposition of this species at 100 and 1000 mg/l (Seenivasagan et al. 2009). Gravid female *Aedes albopictus* are also repelled by n-heneicosane (secreted by *Ae. aegyptis* larvae) at 30, 50, 100 and 200 ppm (Mendki et al. 2000). Also, 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 olfactory 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* (Linneaus) (Duyck et al. 2004) and *Ostrinia furnacalis* (Guenée), *Ostrinia scapulalis* (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.

It is important to note one caveat in interpreting our results that oviposition by *S. calcitrans* was reduced in dung where conspecific and heterospecific larvae had completed their development. In our study, I reared L1 larvae of *S. calcitrans* and *M. domestica* until the pupae stage and I used the rearing substrate for the bioassay. While I suggest a role for chemical cues persisting from larvae or their frass, it may be possible that lower oviposition on a used substrate is also affected by physicochemical properties of these substrates. In comparison with unused substrates, those previously occupied by *S. calcitrans* or *M. domestica* larvae may be less nutritious, have lower water content, and consequently be unsuitable for oviposition. I have shown previously that gravid female *S. calcitrans* oviposit on donkey and sheep dung, which have high nitrogen, phosphorous, potassium and zinc concentrations, in preference over camel and cow dung, which have lower concentrations of the same nutrients (Baleba et al. 2019). In the same study, optimal developmental time was associated with moderate dung water content, which was characteristic of the preferred dung types. In *Junonia coenia* (Hübner) gravid females prefer host-plants with high nitrogen levels (Prudic et al. 2005). To overcome this potential limitation, further studies are required with an additional treatment of weathered dung in which no larvae have developed.

I 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 (Duyck et al. 2004; 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* (Linneaus). In darkness, I observed that gravid female *S. calcitrans* deposited the same number of eggs on substrates with and without mites. This indicates that rather than olfactory 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 colonisation by con- and heterospecific larvae, avoidance of *M. muscaedomesticae* also benefited the fitness of *S. calcitrans* offspring. I found that egg hatchability, and larval and adult survival were higher in substrates without the mite *M. muscaedomesticae*. Axtell (1963) 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. I 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*.

3.6 CONCLUSION

In summary, our results indicate that gravid female *S. calcitrans* avoid substrates colonised 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. I believe that the avoidance of substrates with conspecific and heterospecific larvae in *S. calcitrans* is mediated by olfactory cues. Therefore, I recommend that additional studies be conducted to identify the chemical cues implicated in this avoidance behaviour and their source. This will aid in development of improved control strategies against *S. calcitrans*.

CHAPTER 4 Effect of larval density and substrate quality on the wing geometry of Stomoxys calcitrans L. (Diptera: Muscidae)

This chapter is published as:

Baleba, S. B. S., Torto, B., Masiga, D., Weldon, C. W. and Getahun, M. N. Effect of larval density and substrate quality on the wing geometry of *Stomoxys calcitrans* L. (Diptera: Muscidae). *Parasites and Vectors*. **12**:222

4.1 ABSTRACT

In insects, oviposition decisions may lead to egg deposition in substrates with different larval density and nutritional levels. Individuals developing in such substrates may present plasticity in their phenotype. Here, I investigated the effect of two factors related to oviposition decisions, namely larval density and substrate quality, on the wing size and wing shape of the stable fly, *Stomoxys calcitrans* L. (Diptera: Muscidae). I reared *S. calcitrans* larvae at different densities (5, 15 and 25) and on different substrates (camel, cow, donkey and sheep dung). For each fly that emerged, I recorded body weight, and detached, slide-mounted and photographed the right wing. Next, I collected 15 landmarks on each photographed wing, and applied geometric morphometric analysis to assess variation in wing size and wing shape of *S. calcitrans* across the different larval densities and substrate types. I observed that wing size and wing shape of *S. calcitrans* were affected by larval density and the nature of the developmental substrate. Flies reared in a group of 5 had larger wing centroid size, wing length, wing width, wing area and wing loading compared with those reared in a group of 25. Also, flies developed in donkey and sheep dung had larger wing centroid size, wing length, wing width, wing area and wing loading in comparison with those grown in camel and cow dung. Canonical variate analysis followed by discriminant analysis revealed significant wing shape variation in *S. calcitrans* across the different densities and substrates. Wing size had a significant but weak positive effect on wing shape. This study demonstrates the high sensitivity of *S. calcitrans* wings to variation in larval density and developmental substrate, and that use of landmark-based geometric morphometric analysis could improve our understanding of how flies of veterinary importance respond to environmental variability

4.2 INTRODUCTION

In holometabolous insects, individual fitness mostly relies on oviposition decisions by gravid females. When, where and how mothers deposit their eggs can affect the performance and phenotype of their progeny (Mousseau 1998). Therefore, it is important for females to oviposit on a substrate that provides the best conditions for the next generation. However, a female may fail to make the seemingly optimal choice to oviposit on an appropriate substrate that could enhance offspring fitness. For instance, Heard (1994) found that in the pitcher plant mosquito, *Wyeomyia smithii* Coquillett, although larval fitness is better in pitchers with fewer conspecific and more midge larvae, gravid females did not deposit more eggs in such pitchers. Instead, they laid more eggs in pitchers containing either midges or conspecific larvae. Wong et al. (2012) found that larval survival and development of *Aedes aegypti* L. was poor in containers where gravid females laid more eggs. This imperfection in oviposition decisions generally leads to phenotypic variation (Wolf and Wade 2009; Woestmann and Saastamoinen 2016), in which individuals react to the inputs of their breeding substrate with a change in their form, state, movement, or rate of activity (West-Eberhard 2005). These inputs include environmental factors such as the dietary value of the substrate and the number of individuals sharing the same substrate (density) (Dogan et al. 2016). In evolutionary ecology, understanding how these factors influence organism phenotype is a fundamental concern because such flexibility can affect fitness, generate novelty, facilitate evolution, and structure ecological communities (Whitman and Agrawal, 2009).

Insect wings are good indicators of population responses to changes that occur in their environment (Johansson et al. 2009). Lin et al. (2018) demonstrated that variation in food nutrient content and density are key ecological factors related to the expression of condition-dependent, adaptive phenotypes such as wing polyphenisms. They found that in the brown planthopper, *Nilaparvata lugens* Stål, a serious rice pest, an increase of long-winged *N. lugens* in a population is related to higher glucose levels in host rice plants. Conversely, the appearance of the short-winged form of *N. lugens* is linked to a reduction in host glucose level. Diptera are particularly well suited for studying phenotypic changes induced by the environment because their wings are highly plastic and wing landmarks are homologous across various species (Fraimout et al. 2018). Variation in food quality and population density are key factors associated with fly wing polyphenisms. In *Drosophila buzzatii* Patterson and Wheeler and *Drosophila koepferae* Soto et al. (2008) detected significant differences in wing size and shape between flies that were reared on different cactus hosts.

In *Ae. aegypti*, males and females have longer wings when developed in conditions of low larval density (Jong et al. 2017).

Changes in wing morphology are known to affect insect flight aerodynamics. Long and slender wings are optimal for long-duration flight, while short and broad wings are optimal for slow and agile flight (DeVries et al. 2010). Also, broad wing bases allow a wider range of speed and a narrow wing tip allows less costly, extensive flight (Betts and Wootton 1988). As a consequence, wing morphology is closely related to several insect behavioural activities including food searching, location of breeding sites and sexual partners, and avoidance of natural enemies. In some mosquito species such as *Anopheles gambiae* Giles where wing size is positively correlated with body size, an increase in wing size augments the frequency of blood meals (Takken et al. 1998). This then leads to an increase in the likelihood of pathogen dissemination (Takken et al. 1998). In *Aedes albopictus* Skuse, there is a positive correlation between wing length, larval diet quality, and the number of eggs laid (Yoshioka et al. 2012). It has been demonstrated that males of the olive fruit fly, *Bactrocera oleae* Rossi, with large wings (characterised by a high vibration frequency) achieve higher mating success than males with smaller wings (Benelli et al. 2016).

Fly wing vein networks are excellent models for statistical analysis of size and shape variation (Trotta et al. 2014). In recent years, landmark-based geometric morphometric analysis has been increasingly used to analyse insect wings to address intraspecific variation (Motoki et al. 2012; Gómez et al. 2014), interspecific variation (Wilke et al. 2016; Changbunjong et al. 2016), sexual dimorphism (Vidal and Suesdek 2012; Virginio et al. 2015), parasite detection (Yeap et al. 2014a; Sendaydiego and Demayo), laboratory strain separation (Kitthawee and Rungsri 2011) and phenotypic plasticity (Soto et al. 2008; Sandoval Ramirez et al. 2015; Alves et al. 2016). Geometric morphometric techniques are potent tools to assess the correlation between the size and shape of organisms and environmental variables. The approach uses coordinates of identified morphological "landmarks" to study the form of biological structures in two or three dimensions. It involves several statistical techniques that preserve shape information and detect even subtle morphological variations (Viscosi and Cardini 2011). Moreover, geometric morphometric techniques are cheap, simple and fast (Lorenz et al. 2017). Using geometric morphometric analysis, this study examined the changes that occur in the wings of the stable fly, *Stomoxys*

calictrans L. (Diptera: Muscidae) reared on different substrates and over a range of larval densities.

Stomoxys calcitrans is a cosmopolitan haematophagous fly that mechanically transmits viruses (e.g., West Nile fever virus, Rift Valley fever virus), bacteria (e,g., *Bacillus anthracis, Pasteurella multocida*), protozoans (e.g., *Trypanosoma evansi, Besnoitia besnoit*), and helminths (e.g., *Habronema microstoma, Dirofilaria repens*) to their hosts, which include cattle, camels, horses, dogs, and humans (Lehane 2005; Pitzer et al. 2011; Baldacchino et al. 2013). During outbreaks, *S. calcitrans* can reduce weight gain in cattle by up to 19%, and lead to a 40-60% reduction in milk yields (Carn 1996; Walker 1990). In the USA, Taylor et al. (2012) estimated economic losses attributable to *S. calcitrans* infestation at around $2.211 billion per year. Gravid female *S. calcitrans* oviposit on vertebrate herbivore dung including that of camel, cow, donkey, and sheep, with the latter two the most preferred (Baleba et al. 2019). It has already been demonstrated that the fitness of *S. calcitrans* immature stages (hatchability, developmental time, emergence time, larval and pupal weight) varies across these substrates due to differences in their physicochemical composition (Baleba et al. 2019). However, the way in which preferred and non-preferred substrates affect *S. calcitrans* wing size and wing shape remains unclear. Furthermore, not only the substrate nutrient quality but also larval density should be assessed. I hypothesised that larval density and vertebrate herbivore dung type on which *S. calcitrans* develop would affect wing size and shape.

4.3 MATERIAL AND METHODS

Biological material

Stomoxys calcitrans flies were obtained from a single culture that had been established for approximately 8 months at the Duduville campus of the International Centre for Insect Physiology and Ecology (icipe) in Nairobi (1° 13' 12" S, 36° 52' 48" E; ≈ 1600 m above sea level). By sourcing experimental flies from a laboratory culture, I minimised potential variation between populations. Adults reared from rabbit faeces were kept in cages (75×60×45 cm) under conditions of 25±5ºC and 65±5% relative humidity with a photoperiod of 12L:12D. Flies were fed twice per day (0800 and 1600 hours) on defibrinated bovine blood poured on moistened cotton.

Density experiment

Gravid female *S. calcitrans* from the established colony were allowed to oviposit on donkey dung placed in plastic containers (21.5×14.5×7.4 cm). Baleba et al. (2019) demonstrated that this dung is best for *S. calcitrans* development. To assess the effect of density of *S. calcitrans* on wing size and shape, I reared *S. calcitrans* larvae at varying densities by gently transferring (using soft forceps) 5, 15, and 25 first instar larvae to plastic cups (200 ml) filled with 25 g of donkey dung. I replicated this process several times to obtain 30 emerged females, and 30 emerged males. After emergence, each individual was weighed, killed in 70% ethanol, and its right wing was gently removed from the thorax using a fine clamp. The removed wings were slide-mounted (dorsally placed between two microscope slides) to avoid deformation and to enhance accuracy during photography and landmark collections (Suesdek and Lorenz 2013). I photographed the wings at 16× magnification with a Leica DFC320 digital camera coupled to a Leica S6 microscope.

Substrate quality experiment

To test the effect of substrate on *S. calcitrans* wing size and shape, 10 first instar larvae were transferred to and permitted to develop on 25 g of camel, cow, donkey or sheep dung. It has been shown that these substrates differ in their physico-chemical composition and affect development and adult body weight (Baleba et al. 2019). I replicated the process several times to obtain 30 emerged females, and 30 emerged males. After emergence, each individual of *S. calcitrans* was weighed, killed in 70% ethanol, the right wing was removed and slide-mounted, and wings were photographed as described previously.

Wing geometric morphometric analysis

To collect wing landmark coordinates, I opened the digital photographs in ImageJ software (Abràmoff) and generated Cartesian coordinates for 15 wing landmarks (Fig. 4.1). To quantify measurement error relative to the landmark digitalisation, I collected landmarks for the wings of all 30 individuals reared from donkey dung three times. After executing the generalised Procrustes analysis to extract shape information from the data and eliminate differences in orientation, position and isometric size, I then ran analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA) tests to determine if wing size and the wing shape of *S. calcitrans* varied across the three landmark collections (Klingenberg et al. 2002) (Table 4.1).

Four parameters were derived to describe wing size of *S. calcitrans*: (1) centroid size, (2) wing length (distance between the 6^{th} and 12^{th} landmark), (3) wing width (distance between the 3^{rd} and 8^{th} landmark) (Fig. 4.1) and (4) wing area. The centroid size, also called the "configuration barycentre" is a global size (or multidimensional measurement) calculated as the square root of the sum of squared Euclidean distances between each landmark and the wing centroid. I computed centroid size using PAST software V.3.09 (Hammer et al. 2001). Also, based on the adult weight parameter, I calculated wing loading (in kg/m^2) using the formula: wl = mass/wing area (Ribak et al. 2017). To assess the effect of larval density (5, 15, 25) and substrate type (camel, cow, donkey and sheep dung) on the parameters described above, I ran analyses of variance (ANOVA) followed by post-hoc Student-Newman-Keuls (SNK) tests after checking the wing size parameters for normality using the Shapiro-Wilk test ($P > 0.05$). To identify correlations between centroid size, wing length, wing width, wing area, adult weight and wing loading, I performed separate principal components analysis (PCA) for larval density and substrate type. I used R version 3.5.1 software (R core team 2018) to compute all statistical analyses.

To assess wing shape variation across the different densities and substrates, I imported the raw landmark Cartesian coordinates into MorphoJ software (Klingenberg 2011). This software was first used to perform a generalised Procrustes analysis to extract shape information from the data and eliminate differences in orientation, position and isometric size. Afterwards, I ran separate multivariate analyses of variance (MANOVA) to compare wing shapes across the different larval densities (5, 15, and 25) and substrates (camel, cow, donkey and sheep dung). Using PAST software, I performed thin plate spline analysis to visualise wing shape deformations. I used canonical variate analysis combined with discriminant analysis to analyse the relative similarities and dissimilarities of the different

wing groups. To determine the significance of pairwise differences in mean shapes, I performed permutation tests (10,000 rounds) with Mahalanobis distances and Procrustes distances. To assess the effect of wing size on wing shape (allometry), I fit a linear regression between the Procrustes coordinates and the centroid size, using a permutation test with 10,000 randomisations. For all of these analyses, I excluded the effect of sex because preliminary analyses indicated that wing shape of females and males did not differ.

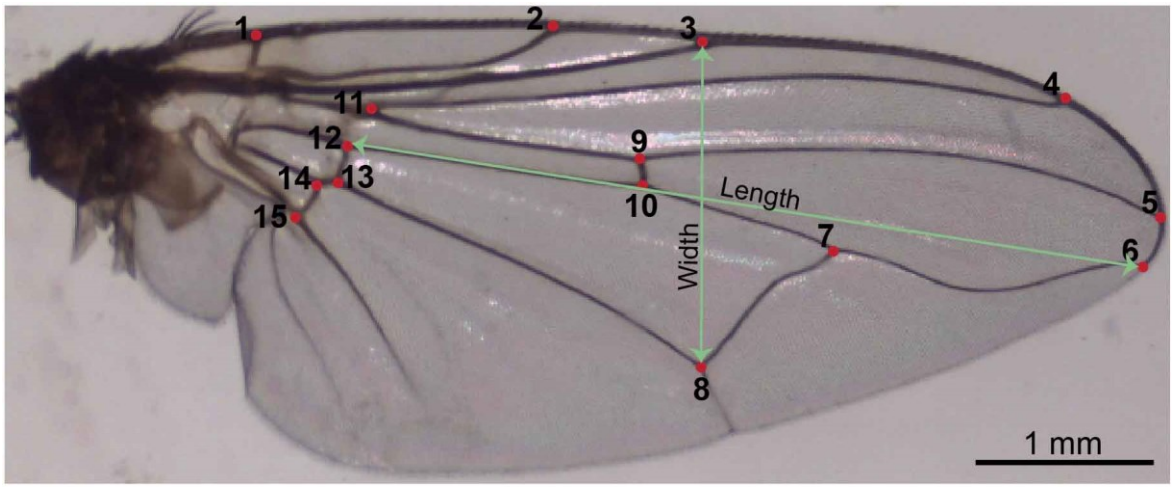

Figure 4.1. Dorsal view of the right wing of *S. calcitrans*. Numbers indicate the location of 15 selected landmarks (described in Table 1).

Table 4.1. Description of the 15 anatomical landmarks used to characterise *S. calcitrans* wing geometry. Numbers relate to landmarks shown in Figure 1.

Anatomic position of landmarks	Description
1	Costal vein intersection with humeral vein
2	Costal vein intersection with subcosta vein
3	Costal vein intersection with radial vein 1
4	Costal vein intersection with radial vein 2+3
5	Costal vein intersection with radial vein 4+5
6	Costal vein intersection Medial vein
7	Basal median cubital vein intersection medial vein
8	Basal median cubital vein and anterior cubital 2
9	Radial vein 4+5 vein intersection with radial medial
10	Medial vein intersection with radial medial vein
11	Radial 2+3 vein intersection with radial 4+5 vein
12	Medial vein intersection with distal median cubital
13	Anterior cubital vein 1 intersection with distal median cubital
14	Anterior cubital vein 1 intersection with anterior cubital vein 2
15	Anal vein 1 intersection with anterior cubital vein 2

4.4 RESULTS

Measurement error test

The landmarks measured repeatedly on the same individual wing (3 times) were not significantly different for both size (ANOVA, $F_{2,84}$=0.03; P=0.97) and shape (MANOVA, $F_{48,2016}$=0.6; Pillai's trace=0.16; P=0.99). Therefore, I ruled out error due to landmark digitalisation, and I considered that any differences found in the morphology of *S. calcitrans* wings resulted from the two factors manipulated in our study (larval density and substrate type).

Effect of larval density on the wing geometry of S. calcitrans

Wing size parameters

Larval density significantly affected wing centroid size ($F_{2,84}$ = 104.1, P<0.0001), wing length ($F_{2,84}$= 97.91, P<0.0001), wing width ($F_{2,84}$= 85.63, P<0.0001), wing area ($F_{2,84}$= 22.67, P<0.0001), adult weight ($F_{2,84}$= 4.51, P= 0.014) and wing loading ($F_{2,84}$= 14.35, P<0.0001) of *S. calcitrans*. I obtained the largest wing centroid size (Fig. 4.2A.i), wing length (Fig. 4.2A.ii), wing width (Fig. 4.2A.iii), wing area (Fig. 4.2A.iv), adult weight (Fig. 4.2A.v) and wing loading (Fig. 4.2A.vi) in flies reared from a group of five larvae. The biplot from the principal component analysis separated flies reared in a group of five from those reared in a group of 25; flies reared in a group of 15 occupied an intermediate position (Fig. 4.2A). The two first dimensions accounted for 85.6 % of total wing size variation. Dimension 1 explained 60.6 % of the entire variation, with wing length as the major contributor. Dimension 2 accounted for 25 % of the total variation, with wing loading as the major contributor. Except for an absence of correlation between adult weight and centroid size (r=0.18, P=0.094), and adult weight and wing area (r=0.02, P=0.83), all other parameters were significantly correlated either negatively or positively (Table 4.2). For instance, wing loading was positively correlated with adult weight (r=0.50, P<0.0001) and negatively correlated with wing width (r= -0.43, P<0.0001), wing length (r= -0.46, P<0.0001), centroid size (r= -0.50, P<0.0001), and wing area (r= -0.60, P<0.0001).

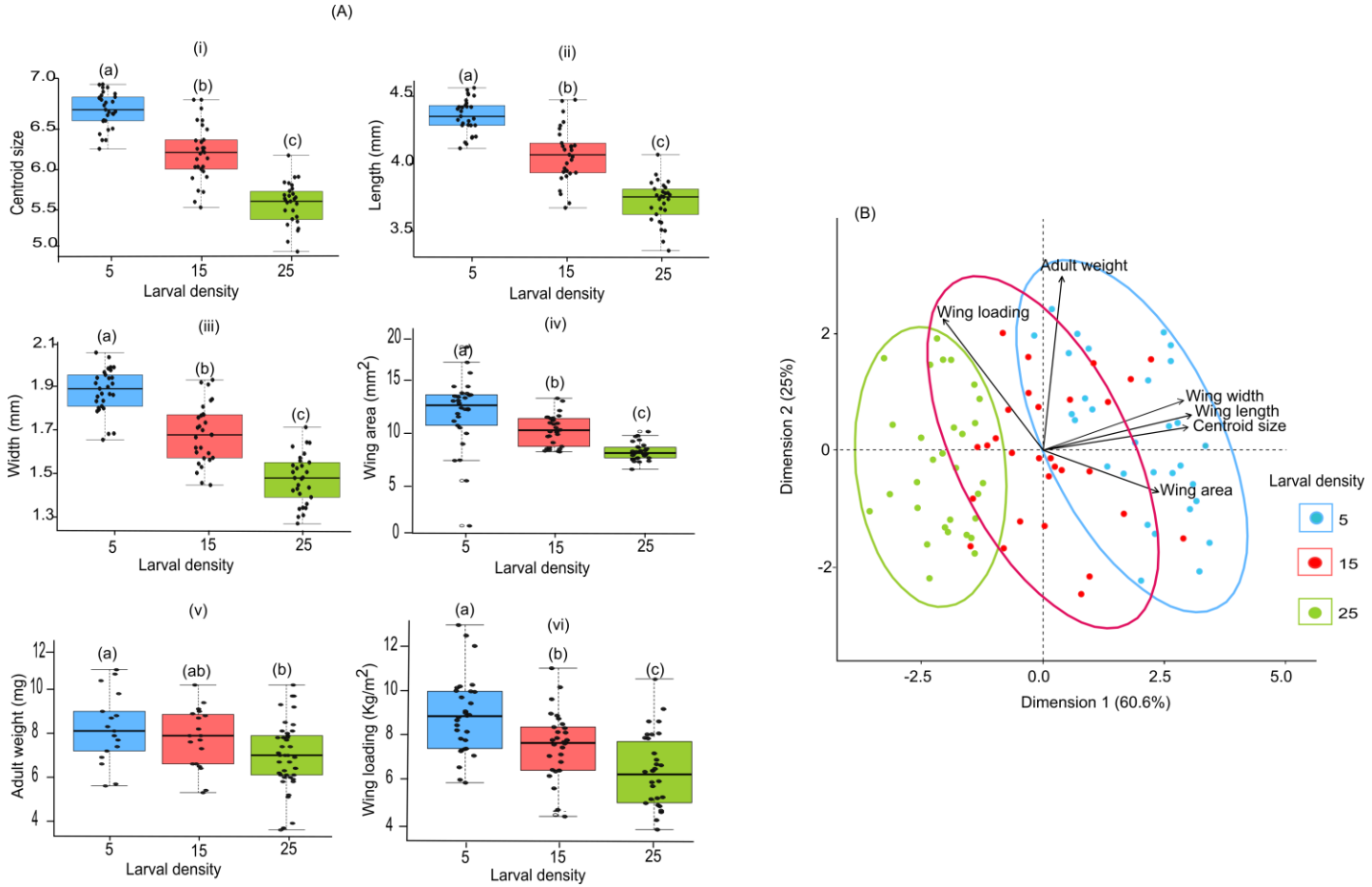

Figure 4.2. Wing size and adult weight of *S. calcitrans* is significantly affected by larval density. (A) Boxplots depicting variation in wing centroid size (i), wing length (ii), wing width (iii), wing area (iv), adult weight (v), and wing loading (vi) across the different larval densities. Boxplot whiskers indicate ±1.5 interquartile range limits. Boxplots with different letters show significant differences as grouped by ANOVA tests followed by SNK

post-hoc tests (*P<0.05,* n=30). (B) Principal component biplot showing the similarities (or dissimilarities) existing among flies reared at different densities (5, 15, and 25).

Table 4.2. Correlation matrix between body weight and wing size parameters of *S. calcitrans* reared at different densities. Values above the diagonal represent p-values from Pearson correlation tests, while values below the diagonal represent correlation coefficient. P-value in bold are not significant.

	Wing area	Adult weight	Wing width	Wing length	Wing loading	Centroid size
Wing area	-	**0.83**	<0.001	<0.001	<0.001	<0.001
Adult weight	0.02	-	0.016	0.024	<0.001	**0.094**
Wing width	0.54	0.25	-	<0.001	<0.001	<0.001
Wing length	0.57	0.24	0.97	-	<0.001	<0.001
Wing loading	-0.6	0.49	-0.43	-0.46	-	<0.001
Centroid size	0.55	0.18	0.89	0.93	-0.49	-

Wing shape parameters

The wing shape of *S. calcitrans* significantly differed between the larval densities (MANOVA, $F_{52,1872}$=2.26; Pillai's trace=0.99; P=0.0059). The thin plate spline (Fig. 4.3A) showed the variation in expansions and contractions in wing vein intersections (landmarks) of flies emerged from densities of 5, 15, and 25. For instance, in flies from densities of 5 and 15, the landmarks 4, 5, and 6 underwent expansion movement, while in the flies from a density of 25, the same landmarks contracted. Additionally, in flies from densities of 5 and 25, expansions in the landmarks 12, 13 and 14 were more pronounced (red coloured) compared to those of flies from a density of 15 (yellow coloured). The landmarks 7, 8, 9 and 10 expanded in wings emerged from a density of 15, while in fly wings from densities of 5 and 25, these landmarks contracted.

Canonical variate analysis discriminated flies emerged from each density based on wing shape (Fig. 4.3B). The two first dimensions accounted for 100 % of the total shape variation (CV1=82.56% and CV2=17.44%), and clustered wing shapes in three distinct groups based on the three larval densities. Pairwise comparisons using discriminant analysis with Mahalanobis distances revealed a highly significant difference in *S. calcitrans* wing shapes (Fig. 4.3C; Table 4.3; permutation test, 10000 replicates, P<0.0001). When Procrustes distances were used, I found that wing shape of flies reared from densities of 15 and 25 were similar (P=0.16). Regression of Procrustes coordinates on centroid size between densities was significant (permutation test with 10000 rounds, P=0.008), with allometry explaining 2.97 % of total shape variation.

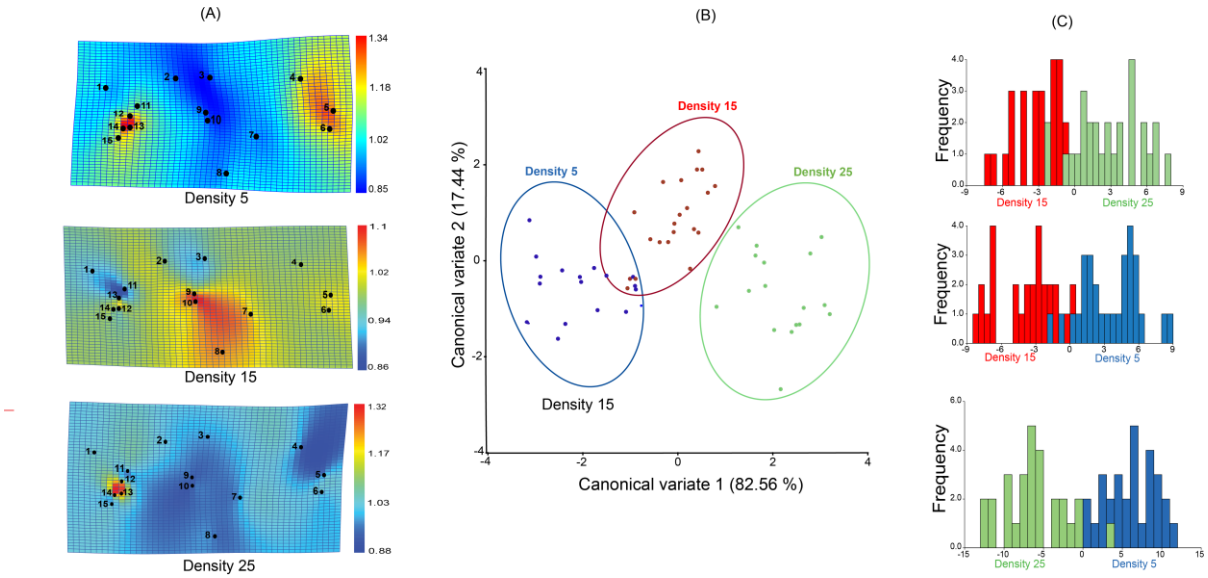

Figure 4.3. Wing shape of *S. calcitrans* is significantly affected by larval density. (A) Thin plate spline deformation grids modelling the difference of *S. calcitrans* wing shape across the different larval densities. The number displayed on each grid represents the landmark positions. Yellow to orange-red colours indicate landmark expansions, light to dark-blue indicates landmark contraction. (B) Scatter plot showing the difference in wing shapes of *S. calcitrans* individuals reared at different larval densities along the first two canonical variate [CV1 (82.56%) and CV2 (17.44%)] axes with 90% confidence ellipses. (C) Discrimination histograms comparing the *S. calcitrans* wing shape between pairs of larval densities.

Table 4.3. Difference in the shape of right wings from *S. calcitrans* reared at a density 5, 15, and 25. P-values (above the diagonal); distances between populations (below the diagonal). *P<0.05* denotes a significant difference.

	Mahalanobis distances			**Procrustes distances**		
	Density 5	Density 15	Density 25	Density 5	Density 15	Density 25
Density 5	___	<0.001	<0.001	___	0.0471	<0.001
Density 15	2.01	___	<0.001	0.0086	___	**0.16**
Density 25	3.38	2.23	__	0.0134	0.0083	__

Effect of developmental substrate on the wing geometry of S. calcitrans

Wing size parameters

Wing centroid size (Fig. 4.4A.i; $F_{3,122}$=39.24; P<0.0001), wing length (Fig. 4.4A.ii; $F_{3,122}$=34.9; P<0.0001), wing width (Fig. 4.4A.iii; $F_{3,122}$=46.98; P<0.0001), wing area (Fig. 4.4A.iv; $F_{3,122}$=31.02; P<0.0001), adult weight (Fig. 4.4A.v; $F_{3,122}$=140.4; P<0.0001) and the wing loading (Fig. 4.4A.vi; $F_{3,122}$=67.79; P<0.0001) of *S. calcitrans* individuals reared from various animal dung differed significantly. All these parameters were highest in flies reared on donkey and sheep dung. The principal component analysis differentiated flies emerged from the different herbivore dung (Fig. 4.4B). The first dimension accounted for 55.5 % of variation in wing shape and were highly correlated with adult weight. The second dimension explained 19.4 % of the total variation and was highly associated with wing length. Other than a lack of correlation between wing loading and wing length (r=0.16, P=0.08), all wing size parameters were positively correlated (Table 4.4).

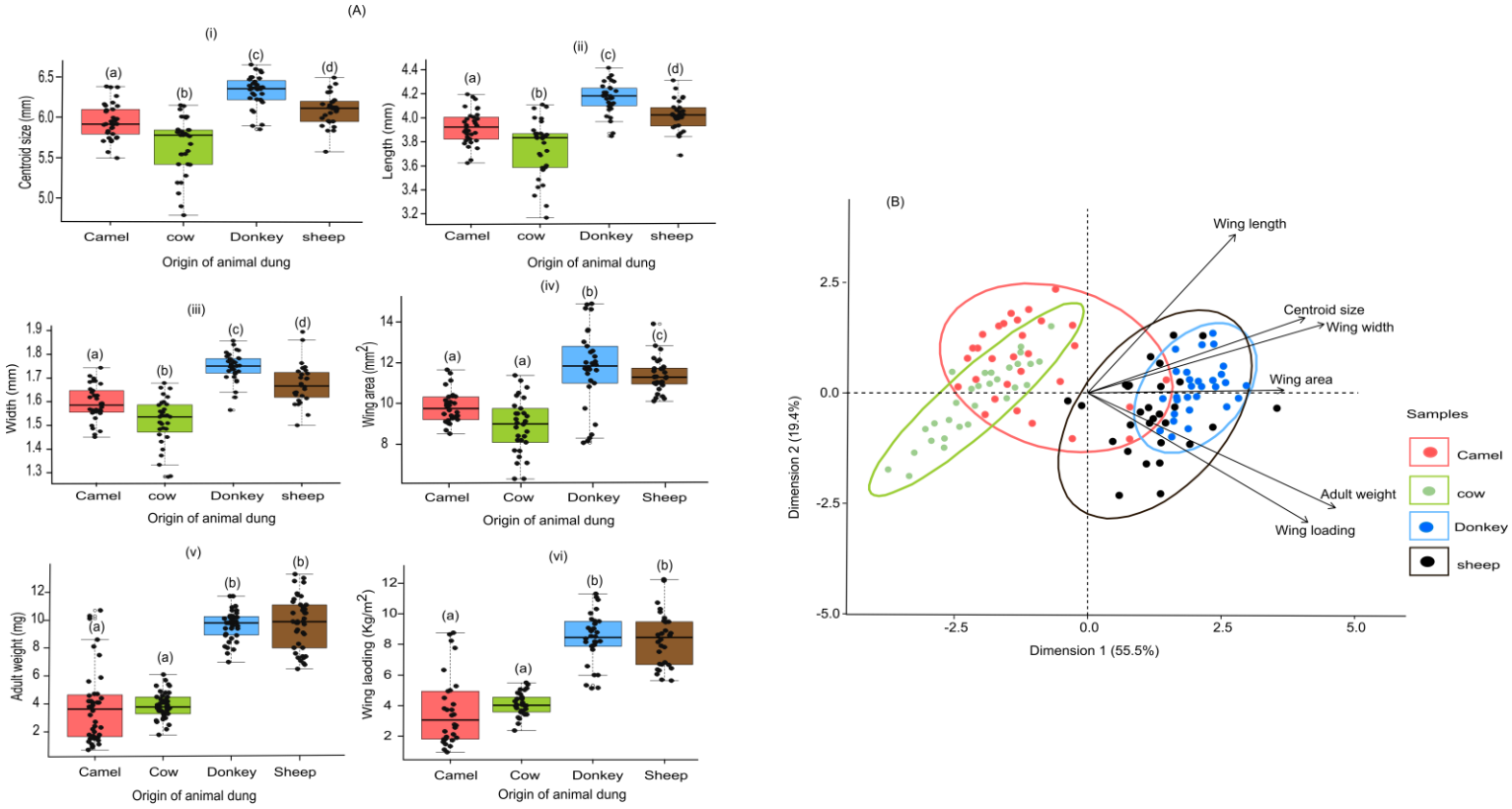

Figure 4.4. Wing size and adult weight of *S. calcitrans* significantly varies across different developmental substrates. (A) Boxplots depicting variation in wing centroid size (i), wing length (ii), wing width (iii), wing area (iv), adult weight (v), and wing loading (vi) across the different dung substrates. The limit of each boxplot whiskers represents the minimum and maximum of all the data. Boxplots with different letters depict significant differences as grouped by ANOVA tests followed by SNK post-hoc tests ($P<0.05$, n=30). (B) Principal component biplot showing the similarities (or dissimilarities) existing among flies reared from different dung types.

Table 4.4. Correlation matrix between body weight and wing size parameters of *S. calcitrans* reared from different developmental substrates. Values above the diagonal represent p-values from Pearson correlation tests, while values below the diagonal represent correlation coefficient. P-value in bold are not significant

	Centroid size	Wing length	Wing width	Wing area	Wing loading	Wing mass
Centroid size	1	<0.001	<0.001	<0.001	<0.001	<0.001
Wing length	0.43	1	<0.001	0.01	**0.08**	0.035
Wing width	0.66	0.52	1	<0.001	<0.001	<0.001
Wing area	0.46	0.23	0.49	1	<0.001	<0.001
Wing loading	0.39	0.16	0.47	0.3	1	<0.001
Wing mass	0.46	0.19	0.54	0.57	0.95	1

Table 4.5. Difference in the shape of right wings from *S. calcitrans* reared from various animal dung. P-values (above the diagonal); distances between populations (below the diagonal). *P<0.05* denotes significant difference

	Mahalanobis distances				**Procrustes distances**			
	Camel dung	Cow dung	Donkey dung	Sheep dung	Camel dung	Cow dung	Donkey dung	Sheep dung
Camel dung	—	<0.0001	<0.0001	<0.0001	—	**0.20**	0.0002	<0.0001
Cow dung	1.7975	—	<0.0001	<0.0001	0.0067	—	<0.0001	<0.0001
Donkey dung	2.2631	2.3678	—	<0.0001	0.0109	0.0125	—	0.0476
Sheep dung	3.0651	3.1064	1.9544	—	0.0155	0.0153	0.0081	—

Wing shape parameter

Developmental substrate significantly affected the wing shape of *S. calcitrans* (MANOVA, $F_{78,3172}$=4.07; Pillai's trace=1.17; P<0.0001). This is clearly illustrated in the thin plate spline deformation grid (Fig. 4.5A). For instance, in flies emerged from camel dung (Fig. 4.5 A.i), landmarks 12, 13, 14 and 15 underwent expansion movement, whereas in flies from cow (Fig. 5 4A.ii), donkey (Fig. 4.5A.iii) and sheep (Fig. 4.5A.iv) dung, the same landmarks contracted. Canonical variate analysis (Fig. 4.5B) and discriminant analysis (Fig 4.5C) separated *S. calcitrans* wing shapes according to the dung in which flies developed. The first two dimensions of the canonical variate analysis explained 85.06 % of the total *S. calcitrans* wing shape variation (Fig. 4.5B; CV1=66.06% and CV2=16%). All pairwise permutation tests performed with Mahalanobis distances revealed that the shape of *S. calcitrans* wings diverged significantly when reared from the different animal dung (Table 4.5; 10000 rounds, P<0.0001). With Procrustes distance estimators, I obtained a non-significant difference in wing shapes only in flies emerged from camel and cow dung (P=0.2). In the allometry test, the centroid size had a significant effect on wing shape (10000 rounds of Permutation tests, P=0.0015), with a variance prediction of 2.45 %.

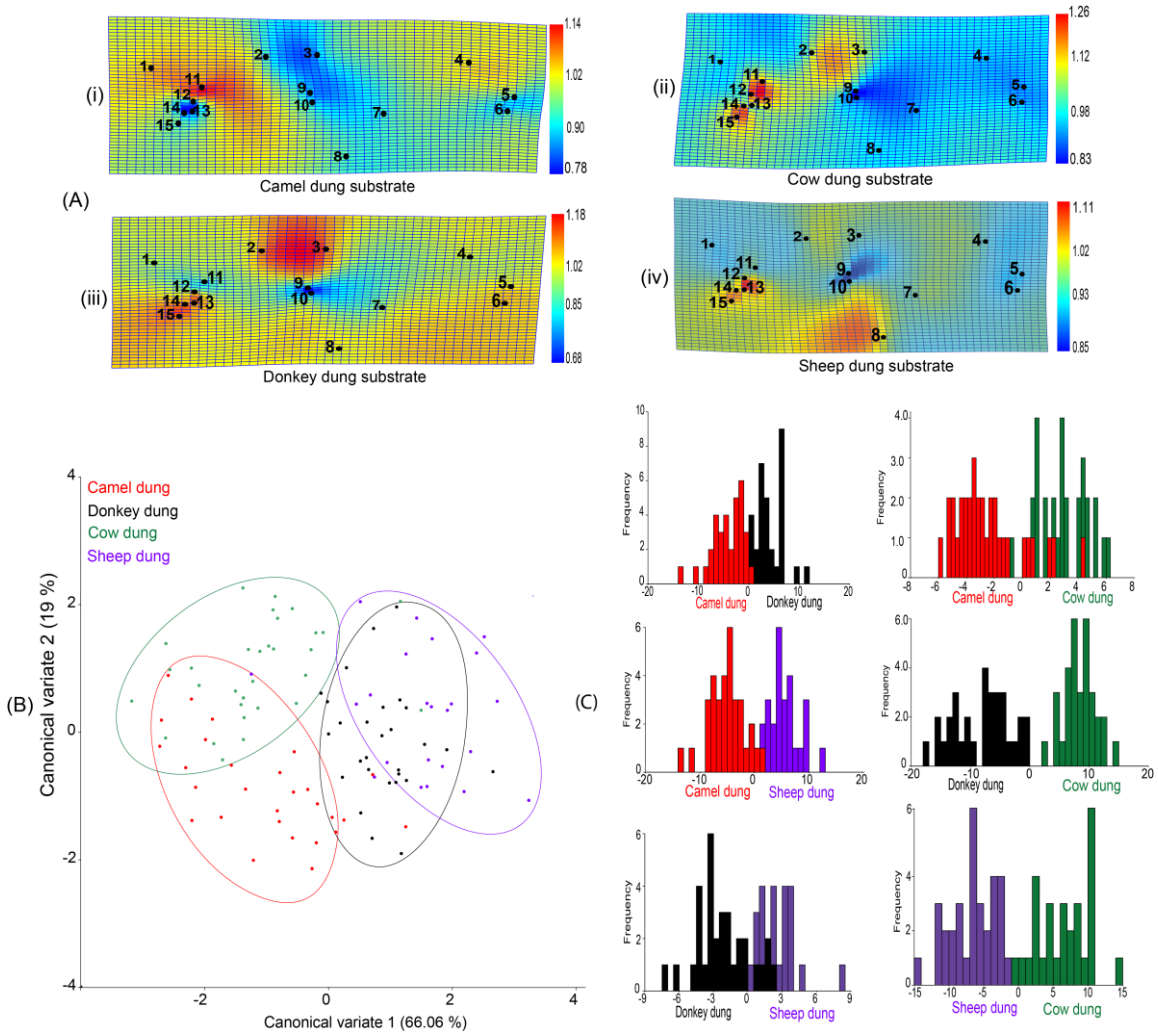

Figure 4.5. The wing shape in *S. calcitrans* is significantly affected by the nature of the developmental substrate. (A) Thin plate spline (TPS) deformation grids modelling the difference of *S. calcitrans* wing shape across the different larval densities. The number displayed on each grid represents the landmark positions. Yellow to orange-red colours indicates landmark expansions, light to dark-blue indicates landmark contraction. (B) Scatter plot showing the difference in wing shapes of *S. calcitrans* individuals emerged from the different larval densities along the first two canonical variates (CV1 (82.56%) and CV2 (17.44%)) axes with 90% confidence ellipses. (C) Discrimination histograms comparing the *S. calcitrans* wing shape between two larval densities.

Table 4.5. Difference in the shape of right wings from *S. calcitrans* reared from various animal dung. P-values (above the diagonal); distances between populations (below the diagonal). *P<0.05* denotes significant difference

	Mahalanobis distances				**Procrustes distances**			
	Camel dung	Cow dung	Donkey dung	Sheep dung	Camel dung	Cow dung	Donkey dung	Sheep dung
Camel dung	__	<0.0001	<0.0001	<0.0001	__	**0.20**	0.0002	<0.0001
Cow dung	1.7975	__	<0.0001	<0.0001	0.0067	__	<0.0001	<0.0001
Donkey dung	2.2631	2.3678	__	<0.0001	0.0109	0.0125	__	0.0476
Sheep dung	3.0651	3.1064	1.9544	__	0.0155	0.0153	0.0081	__

4.5 DISCUSSION

Our study showed that the size and shape of *S. calcitrans* wings exhibit a plastic response to larval density and the quality of the larval substrate. The study also demonstrated the power of the wing landmark-based geometric approach for studying phenotypic plasticity.

I showed that *S. calcitrans* wing size parameters (centroid size, length, and width, area, loading) are affected by larval density (5, 15, and 25) and substrate type (camel, cow, donkey and sheep dung). This indicates the effect of larval developmental conditions on adult wing size. Our study is consistent with previous work on *Ae. aegypti* that revealed the influence of larval density and substrate quality on wing size (Stephens and Juliano 2012). The wing size variation obtained in our study may be due to variability in nutrients. Baleba et al. (2019) previously determined that donkey and sheep dung, from which emerging flies had the largest wing size, had higher concentrations of specific micronutrients (nitrogen, phosphorous, potassium and zinc) in comparison with camel and cow dung. Furthermore, competition for limited nutrients by *S. calcitrans* larvae may also affect wing size. Dutra et al. (2016) found that under high larval density, *Wolbachia*-uninfected *Ae. aegypti* presented reduced wing size (centroid size) and a lower body glucose concentration.

Wing sizes are closely related to the flight capacity in insects. Individuals with longer wings are better at flying compared to those with shorter wings (DeVries et al. 2010). Long wings favour wider variation in speed and long flight duration (Hoffmann et al. 2007). I obtained longer wings in *S. calcitrans* reared in a group of five or when reared from donkey and sheep dung. Long wings allow insects to fly at a great speed for a long period of time and cover a large area (Berwaerts et al. 2002; Davis and Holden 2015). For instance, released *Ae. aegypti* with larger wings are more successful in host-seeking and oviposition site location (Harrington et al. 2005; Kay and Muir 1998). I predict that *S. calcitrans* developed under lower density conditions are more likely to have good flight capacity. Also, developmental substrates such as donkey and sheep dung may increase the efficiency of *S. calcitrans* flight. This result supports Baleba et al. (2019), who found that dung types preferred by female *S. calcitrans* for oviposition were best for offspring growth and development. In this case, preferred substrates lead to potential adult fitness benefits associated with wing size. Low larval density leads to larger adults with long wings due to higher resource availability for growth and nutrient storage in the larval stage. Similarly, development of larvae in preferred

dung types would lead to larger adults with larger wings. Such changes may influence dispersal, mating, and vector competency of *S. calcitrans*, rendering this fly more capable of spreading pathogens.

Our study also showed that larval density and substrate type affected *S. calcitrans* wing shape. The thin-plate spline analyses showed that most of the shape changes (landmark movements) occurred on the radial (landmarks 11, 12, 13, 14 and 15) and medial (2, 3, 7, 8, 9 and 10) portions of the *S. calcitrans* wing. Oguz et al. (2017) observed the same variation in the radial portion of the wing of *Phlebotomus tobbi* Adler and Theodore. Pieterse et al. (2017) found variation in wing landmarks located at the costal, sub-costal and radial veins of *Bactrocera dorsalis* (Hendel) and *Ceratitis capitata* (Wiedemann) reared from nectarine, plum, pear and apple. According to Wootton et al. (2003) and Shimmi et al. (2014), the radial and the medial portions of insect wings play a critical function in the aerodynamics of insect flight. Wootton (1981) suggested such changes may influence the wing strength, beat pattern and ultimately the dispersal potential of a fly. Therefore, the wing shape deformation observed here may affect the flight performance of *S. calcitrans*, their ability to find a host for a blood meal, and consequently, vectorial capacity. Several studies with no emphasis on wing morphology have already demonstrated the indirect effect of larval density and food quality on vector competence. For instance, in *Ae. albopictus,* a greater dissemination rate of Sindbis virus by the adult is the consequence of high levels of competition experienced by the larvae (Alto et al. 2005). In *Anopheles stephensi* Liston, larvae developed in a nutritious substrate are more likely to transmit the human malaria parasite, *Plasmodium falciparum*, than those developed in a substrate with a poor nutritional value (Shapiro et al. 2016). The discriminant factors on which the differentiation between flies reared from different density or dung type was based were not free of some allometric effects. In other words, wing size contributed significantly to wing shape variation. However, in the case of both larval density and developmental substrate, less than 3% of variation in wing shape was attributed to size. Such low residual variation indicates that changes in the relative position of landmarks as wing size increases are minimal (Harrington et al. 2005).

4.6 CONCLUSION

This study highlights the effect of larval density and developmental substrate of wing size and wing shape of *S. calcitrans* using the landmark-based geometric morphometric method. The method satisfactorily discriminated *S. calcitrans* emerged from different larval densities

and substrates based on the size and the shape of their wings. While there was a significant effect of size variation on variation in shape, but this accounted for less than 3% of variation. Future studies on flight performance of *S. calcitrans* as well as their vectorial capacity in pathogen transmission when reared under different larval conditions are required. However, our results demonstrate a role for larval density and developmental substrate to influence wing size and to some extent wing shape, which might have a significant effect on flight and dispersal of adult *S. calcitrans*.

CHAPTER 5 Larval experience of stable fly, *Stomoxys calcitrans* L. 1758 (Diptera: Muscidae) does not influence oviposition preference in gravid females

This chapter is published as:

Baleba, S. B. S., Weldon, C. W., Masiga, D., Torto, B. and Getahun, M. N. Larval experience of stable fly, *Stomoxys calcitrans* Linneaus 1758 (Diptera: Muscidae), does not influence oviposition preference in gravid females. *Ecological Entomology*.

5.1 ABSTRACT

The Hopkins′ host selection principle (HHSP) proposes that, in holometabolous insects, gravid females prefer to oviposit on the substrate on which they developed. In this study, I tested the HHSP using the haematophagous stable fly, *Stomoxys calcitrans* (Diptera: Muscidae). Using no-choice and two-choice tests, I first sought to demonstrate whether *S. calcitrans* larvae recognise their rearing substrate. I found that when a rearing substrate is offered to *S. calcitrans* larvae singly (no-choice) or associated with a non-rearing substrate (two-choice), they were able to recognise the substrate in which they developed. This ability disappeared when larvae were transferred to another substrate for 5 hr. Next, using oviposition bioassays, I elucidated whether information gathered by pre-imaginal stages during their development can persist in the adult stages after metamorphosis. I hypothesised that gravid females emerging from clean and uncleaned pupae reared on sheep and camel dung would still prefer to lay on their rearing substrate. Surprisingly, gravid females did not prefer to oviposit on their developmental substrate. In addition, failure to recognise and prefer their developmental substrate was found whether gravid females had emerged from puparia that were cleaned or contaminated with sheep dung. I conclude that *S. calcitrans* adult preference is not influenced by the experience of their pre-imaginal stages. Overall, the HHSP was not supported by our study, suggesting that this principle may not apply to generalist insects characterised by high plasticity in oviposition substrate acceptance.

5.2 INTRODUCTION

In holometabolous insects, pre-imaginal stages (egg and larva) and the adult stage are different in terms of their body form, life style, and diet. However, it may be possible that information gathered by pre-imaginal stages is retained during metamophosis to be transferred to the adult. Several studies have claimed a direct or indirect effect of larval experience on adult behaviour in different holometabolous groups such as Lepidoptera (Olsson et al. 2006; Proffit et al. 2015; Hu et al. 2018), Diptera (Tully et al. 1994; Ray 1999; Blackiston et al. 2008), Coleoptera (Reh-Hamburg 1924; Rietdorf and Steidle 2002) and Hymenoptera (Lecomte and Thibout 1993; Gandolfi et al. 2003), although others show no support for a connection (Rojas and Wyatt 1999; Kerpel and Moreira 2005; Janz et al. 2009). Three hypotheses have been raised to provide a functional mechanism for information retention across life stages: the "Hopkins' host selection principle" (Hopkins 1916), the "neo-Hopkins principle" (Jaenike 1983) and the "chemical legacy hypothesis" (Corbet 1985). The Hopkins' host selection principle (HHSP) refers to the observation that, in insects, adult stage behaviour is conditioned by experience during their pre-imaginal stages. For the neo-Hopkins principle, adult behaviour is shaped by the experience gained during or shortly after their emergence from the pupae. The chemical legacy hypothesis considers that the connection between larval and adult behaviour is made by the chemical cues bequeathed by the larval stage to the adult stage. In practice, when illustrating the link between larval and adult stage memories, it is necessary to clean and physically separate pupae from chemical cues associated with the larval environment to eliminate the opportunity for habituation or sensitization of the emerging adults (Tully et al. 1994; Corbet 1985; Rietdorf & Steidle 2002; Gandolfi et al. 2003). By doing so, a chemical legacy can be ruled out. Thereafter, any connection between larval and adult experience would result from the transmission of neuronal information from the larva to adult during metamorphosis, enabling persistence in the adult brain of memories formed during the larval stage (Blackiston et al. 2008).

The effect of pre-imaginal conditioning in adult behaviour is well documented in phytophagous insects. Tully et al. (1994), after electroshocking larvae of *Drosophila* in the presence of ethyl acetate or isoamyl acetate, found that the conditional odour avoidance observed in larvae was still present in adults 8 days later. In *Musca domestica*, Ray (1999) showed that adults preferred specific odours to which they were exposed during larval development. Host selection behaviour in adult granary weevils, *Sitophilus granary*, was shaped by their experience during the immature stages (Rietdorf and Steidle 2002). The

onion fly, *Delia antiqua*, preferred to oviposit on their natal host (Ning et al. 2018). In hematophagous insects (mainly in mosquitoes), learning in the larval (Ferrari et al. 2008; Baglan et al. 2017) and adult stage (Menda et al. 2013; Roberts 2014) has already been demonstrated. However, little is known about the possible transmission of information from the larva to adult in hematophagous insects. Therefore, in this study, I examine whether the information gathered by larvae of a blood-feeding insect can reach the adult stage through metamorphosis, using the stable fly, *Stomoxys calcitrans*, as a model.

Stomoxys calcitrans is a cosmopolitan blood-feeding insect that mechanically transmits viruses (e.g., West Nile fever virus, Rift Valley fever virus), bacteria (e,g., *Bacillus anthracis, Pasteurella multocida*), protozoans (e.g., *Trypanosoma evansi, Besnoitia besnoit*), and helminths (e.g., *Habronema microstoma, Dirofilaria repens*) to their adult hosts, which include cattle, camels, horses, dogs, and humans (Lehane 2005; Pitzer et al. 2011; Baldacchino et al. 2013). During outbreaks, *S. calcitrans* can reduce weight gain in cattle by up to 19%, and lead to a 40-60% reduction in milk yield (Carn 1996; Walker 1990). In the USA, Taylor et al. (2012) estimated economic losses attributable to *S. calcitrans* infestation at around $2.2 billion per year. Like all dipteran species, *S. calcitrans* has a holometabolous life cycle. Eggs are laid and the larval (with 3 instars) and pupal stages are completed in the dung of herbivorous animals such as cows, donkeys, horses, goats, and sheep (Hafez & Gamal-Eddin 1959, Jeanbourquin & Guerin 2007; Salem 2012; Cançado et al. 2013). In this study, I investigated whether experience gained by larvae of *S. calcitrans* throughout their development influenced female oviposition decisions. Specifically, I determined the ability of *S. calcitrans* larvae to recognise their rearing substrate and established whether recognition of the rearing substrate was maintained for 5 hr. Finally, I assessed whether rearing substrate recognition persisted in gravid females.

5.3 MATERIAL AND METHODS

Test insects

Stomoxys calcitrans flies were obtained from a culture established at the Duduville campus of the International Centre of Insect Physiology and Ecology (*icipe*) in Nairobi (1° 13' 12" S, 36° 52' 48" E; ≈ 1,600 m above sea level). Adults reared from rabbit dung were kept in cages (75×60×45 cm) under buffered conditions of 25±5^{o}C and 65±5% relative humidity with a photoperiod of 12L:12D. Flies were fed twice per day (0800 and 1600 hr) on defibrinated bovine blood (from slaughter houses) poured on moistened cotton. When adults were sexually mature, camel and sheep dung placed in the plastic container (21.5×14.5×7.4 cm), were presented to gravid females for oviposition. After a period of 24 hr, each substrate was removed and transferred to another cage. Larval development was monitored daily until the third larval instar (L3) was reached. This developmental stage was identified by inspecting the posterior spiracles, which are characterised by triangular discs with three sinuous slits (Friesen et al. 2017).

Experience test

To establish whether *S. calcitrans* larvae change their behaviour in relation to experience, I hypothesised that L3 larvae of *S. calcitrans* reared on a specific substrate would be attracted to the same substrate. To test this hypothesis, I began with a "no-choice" test (Fig 5.1A) where L3 larvae reared on camel dung (Fig. 5.1A.i) or sheep dung (Fig. 5.1A.ii) were gently placed away from either substrate in an arena (described below) using soft forceps. This was done to confirm that larvae were capable of relocating the substrate within an arena. Afterwards, I performed a series of "two-choice" tests. For the first two-choice test (Fig 1B), L3 larvae reared on camel dung (Fig. 5.1B.i) or sheep dung (Fig. 5.1B.ii) were permitted to choose between camel and sheep dung in an arena. I established that L3 larvae preferred their rearing substrate over non-rearing substrate. To elucidate whether this behaviour was the result of innate preference for particular dung types, I hypothesized that if L3 larvae were presented with two unfamiliar substrates, they would exhibit no preference for either substrate. To test this, I reared L3 larvae on camel dung and exposed them to rabbit and sheep dung (Fig 5.1C.i). I also reared L3 larvae on sheep dung and exposed them to camel and rabbit dung (Fig 5.1C.ii).

For all tests, I used plastic containers (21.5×14.5×7.4 cm) as a choice arena in which 80 larvae were released one by one in the middle of the container (7.5 cm away from the tested substrates). The amount of substrate in each test arena was 50 g. Ten minutes after setting-up each replicate, larvae found on a substrate were considered to have made a choice. The duration of the tests was selected based on the results of preliminary bioassays where larvae were given 5, 10, 15, and 20 min to move to a substrate. The number of larvae moving to a particular substrate did not improve significantly (10 min: 57(71.25%); 20 min: 62 (77.5%), n=80, χ^2=0.21, d.f=1, *P=0.65*) if larvae were given longer than 10 minutes (data not presented). Larvae that were still in the middle of the container were recorded as not having made a choice. The substrate position was changed between replicates. All tests were conducted in darkness because *S. calcitrans* larvae exhibit negative phototaxis. After each test, containers were cleaned with distilled water and tissue paper to avoid retention of odour from dung. I did not use organic solvents because they could be toxic to the larvae or represented a source of contamination. During all the bioassays, I used camel and sheep dung as rearing substrate based on the difference in their physicochemical properties (Baleba et al. 2019)

Experience duration test

In the previous bioassays, I showed that *S. calcitrans* larvae preferred their substrate of development. Here I sought to determine how long this preference was retained. I hypothesised that L3 larvae would progressively lose their aptitude to recognise their rearing substrate when staying in another substrate. To test this, I transferred L3 larvae reared on camel dung to sheep dung for 1 hr (Fig. 5.2A.i), 5 hr (Fig. 5.2B.i), and 24 (Fig. 5.2C.i). Thereafter, I allowed individuals from each treatment to choose between camel and sheep dung in two-choice tests (as described above) to see if they still preferred their rearing substrate. I did the same for L3 larvae reared on sheep dung by transferring them to camel dung for 1 hr (Fig. 5.2A.ii), 5 hr (Fig. 5.2B.ii), and 24 hr (Fig. 5.2C.ii). I selected the different novel dung exposure times based on the review by Margulies *et al.*, (2005), which differentiated among three types of memory in *Drosophila*: a short- (1 hr), middle- (5 hr) and long-term (24 hr) memory.

Experience recovery test

The preceding experiment revealed that after sojourning in another dung type for 5 and 24 hr *S. calcitrans* larvae lose their preference for the initial rearing substrate. I aimed to test if this loss of preference could be be recovered. To do so, I transferred L3 larvae reared on camel dung to sheep dung for 24 hr, then returned them to camel dung for a further 24 hr (Fig. 5.2C). After returning larvae to their original substrate, I allowed them to choose between camel and sheep dung in two-choice tests. Again, the reciprocal procedure was applied to L3 larvae reared on sheep dung (Fig. 5.2D). As in the previous experiment, 80 L3 larvae were released individually in the middle of the arena, and their position was recorded after 10 min of exposure in darkness.

Hopkins' host selection principle and chemical legacy hypothesis tests

HHSP Test

I demonstrated that *S. calcitrans* larvae recognise their rearing substrate. According to the Hopkins' host selection principle, this experience should persist in *S. calcitrans* adults. Therefore, I predicted that gravid females of *S. calcitrans* reared on sheep or on camel dung would still prefer to lay on their rearing substrate. To test this, I reared *S. calcitrans* separately on sheep and camel dung, but I treated their pupae in two ways. In the first treatment, I left pupae in contact with their rearing substrate so that emerging adults were exposed to the chemical cues from the substrate. For the second treatment, I prevented contact of emerging adults with their substrate following the procedure described by Liu & Liu (2006). Briefly, I removed pupae from the substrate, gently cleaned them with distilled water and tissue paper, and transferred them to a sterilised cage (34×34×34 cm) for adult emergence. Once emerged, adults were fed with defibrinated bovine blood poured on wet cotton and kept on this feeding regime until females were gravid (Fig 5.4A.i, 5.4B.i). The obtained gravid females were used in the oviposition tests (Fig 5.4A.ii, 5.4B.ii) as follows. Ten gravid females were transferred to a cage (34×34×34 cm) before being presented with 30 g of oviposition substrate in a Petri dish (diameter: 5.5 cm). Gravid females from sheep dung were presented donkey and sheep dung, while gravid females from camel dung where presented camel and sheep dung. The number of eggs deposited on each substrate was counted after 24 hr. Tests using each treatment were replicated 10 times and a new group of flies was used in each replicate.

Chemical legacy hypothesis test

According to this hypothesis, the traces of chemical cues on the surface of pupae could influence adult oviposition substrate preference. To test this, I reared *S. calcitrans* on rabbit dung until the pupal stage (Fig 5.5A and 5.5B) and I divided the obtained pupae into two groups. I maintained the first group of pupae on rabbit dung until adult emergence (Fig. 5.5A.i), while I cleaned the second group of pupae as described above and transferred them to sheep dung for contamination until adult emergence (Fig 5.5B.i). Once emerged, I fed adults with defibrinated bovine blood. Ten gravid females were used in the oviposition bioassay (Fig 5.5A.ii and Fig 5.5B.ii) with a choice of rabbit and sheep dung provided to them in separate Petri dishes. The number of eggs laid on each substrate was counted after 24 hr. Each test was replicated 10 times and a new group of flies was used in each replicate.

Data analysis

All statistical analyses were performed using R. 3.5.0 (R Core Team 2018). For the no-choice test, I compared the proportion of larvae found in dung to those that were unable to join the dung after 10 min of exposure. For choice tests, I compared the proportion of larvae that had chosen between two types of exposed dung. In both no-choice and choice tests, I used the two-tailed binomial test (McDonald 2009). For the oviposition bioassay, since the collected data were not normally distributed, I used the non-parametric Wilcoxon–Mann–Whitney test to compare the number of eggs laid on different substrates (Dalgaard 2008).

5.4 RESULTS

Experience test

In the "no-choice" test, where 80 larvae were placed individually away from their rearing substrate (Fig 5.1A), a significant majority of larvae reared from camel dung (Fig. 5.1A.i) returned to it (binomial test: 68.8% success, 31.3% failure; p= 0.001). Likewise, a significant majority of larvae from sheep dung (Fig. 5.1A.ii) found their rearing substrate (binomial test: 62.5% success, 37.5% failure; p= 0.033). The remaining larvae from camel and sheep dung did not reach their substrate after 10 minutes.

For the first "two-choice" test (Fig 5.1B), when offering a choice to 80 larvae between their rearing substrate and a non-rearing substrate, I found that in larvae reared on camel dung (Fig. 5.1B.i), five larvae did not make a choice (6.35% non-response). For the 75 remaining larvae, significantly more selected their rearing substrate (camel dung) while the remainder went to sheep dung (binomial test: 62.7 % success, 37.3% failure; p= 0.037). For the larvae reared on sheep dung (Fig. 5.1B.ii), all 80 tested larvae made a choice (0% of non-response). I found that a significant majority selected sheep dung over camel dung (binomial test: 65% success, 35% failure; p= 0.010).

For the second "two-choice test" bioassay (Fig. 5.1C) where I provided larvae with a choice between two non-rearing substrates, they did not exhibit any preference. Of the 80 larvae reared on camel dung and offered rabbit or sheep dung (Fig. 5.1C.i), of those that reached either substrate, the proportion of larvae in rabbit (42.5%) or sheep dung (31.3%) did not differ from random (binomial test: p= 0.141). Also, for the larvae reared on sheep dung (Fig. 5.1C.ii), the proportion of larvae on rabbit dung (52.5%) or camel dung (42.5%) did not differ significantly (binomial test: p= 0.26).

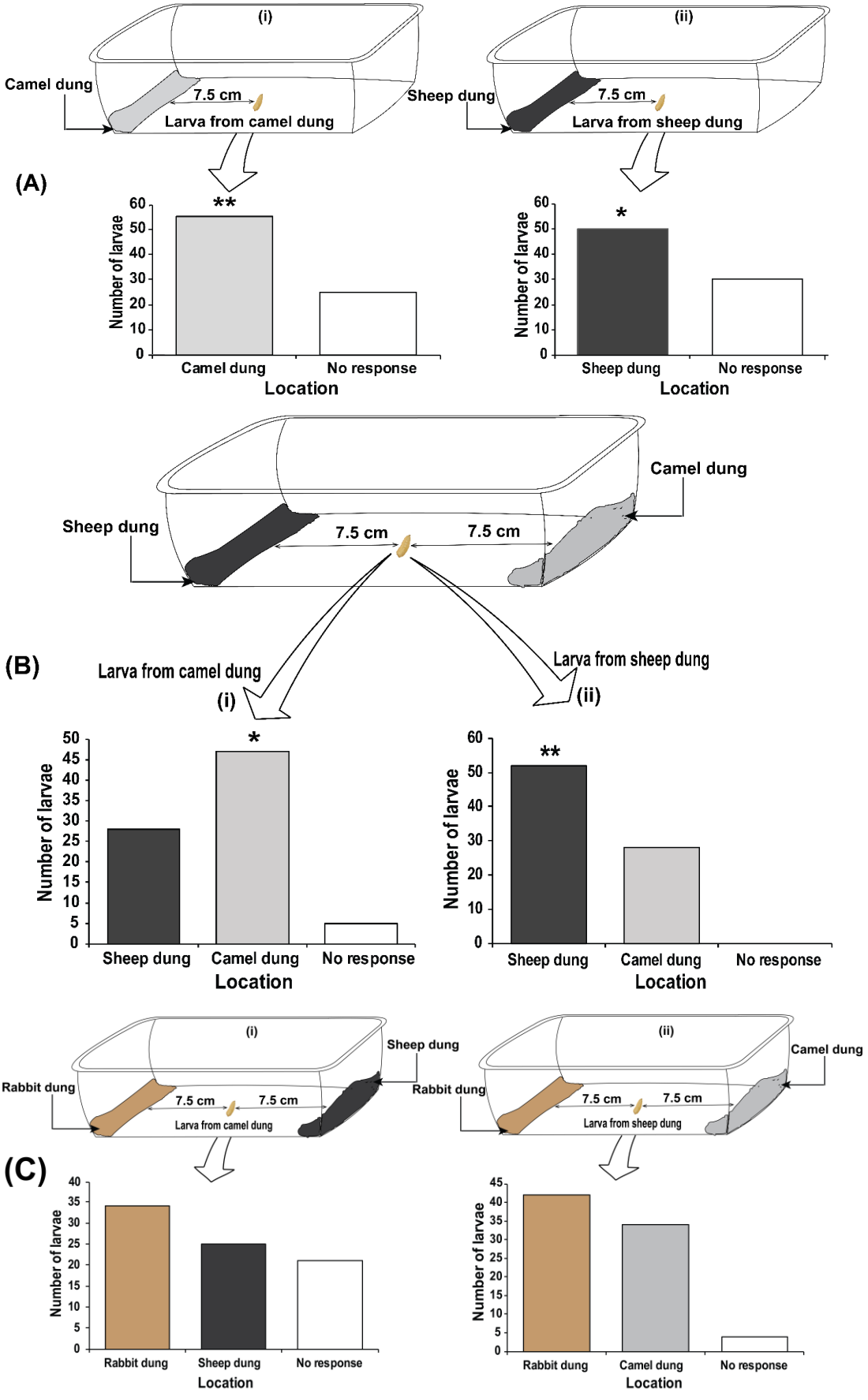
(A)
(i)
Camel dung
7.5 cm
Larva from camel dung
(ii)
Sheep dung
7.5 cm
Larva from sheep dung
Number of larvae
Camel dung
No response
Location
Sheep dung
No response
Location
(B)
Sheep dung
Camel dung
7.5 cm
7.5 cm
Larva from camel dung
(i)
Larva from sheep dung
(ii)
Number of larvae
Sheep dung
Camel dung
No response
Location
Number of larvae
Sheep dung
Camel dung
No response
Location
(C)
(i)
Sheep dung
Rabbit dung
7.5 cm
7.5 cm
Larva from camel dung
(ii)
Camel dung
Rabbit dung
7.5 cm
7.5 cm
Larva from sheep dung
Number of larvae
Rabbit dung
Sheep dung
No response
Location
Number of larvae
Rabbit dung
Camel dung
No response
Location

Figure 5.1. *Stomoxys calcitrans* larvae recognise the substrate in which they developed. (A) No-choice test with third-instar (L3) larvae reared on camel dung (i) or sheep dung (ii) (n= 80). (B) Two-choice test with rearing and non-rearing substrates of camel and sheep dung: L3 larvae reared from camel dung (i) or sheep dung (ii) (n= 80). (C) Two-choice test with two non-rearing substrates: (i) L3 larvae from camel dung given a choice between rabbit and sheep dung; (ii) L3 larvae from sheep dung given a choice between rabbit and camel dung. Asterisks indicate proportions that differed significantly from random (50%) determined by a two-tailed binomial test: * $p<0.05$, ** $p<0.01$.

Experience duration test

Results from the experience duration test show that the ability of *S. calcitrans* larvae to recognise their rearing substrate lasts for up to one hour. Larvae reared on camel dung and transferred on sheep dung for one hour recognised their rearing substrate (Fig 5.2A.i; binomial calculation: 63.0% success, 36.9% success, $p=0.034$). Similar results were found for larvae reared on sheep dung and transferred to camel dung for one hour (Fig 5.2A.ii; binomial calculation: 71.4% success, 28.6% success, $p<0.001$). After sojourning on another substrate for 5 hours, larvae reared on camel dung (Fig 5.2B.i; binomial calculation: 55.4% success, 44.6% failure, $p=0.275$) and sheep dung (Fig 5.2B.ii; binomial calculation: 55.4% success, 48.6% failure, $p=0.910$) did not recognise their rearing substrate. This loss of experience was also evident in larvae that spent 24 hr in another substrate. Larvae reared on camel dung after sojourning on sheep dung for 24 hr did not discriminate between camel or sheep dung (Fig. 5.2C.i; binomial calculation: 53.9% success, 46.1% failure; $p= 0.417$). Similarly, larvae from sheep dung that spent 24 hours on donkey dung did not differentiate between sheep or camel dung (Fig. 5.2C.ii; binomial calculation: 42.7% success, 57.3% failure; $p= 0.103$).

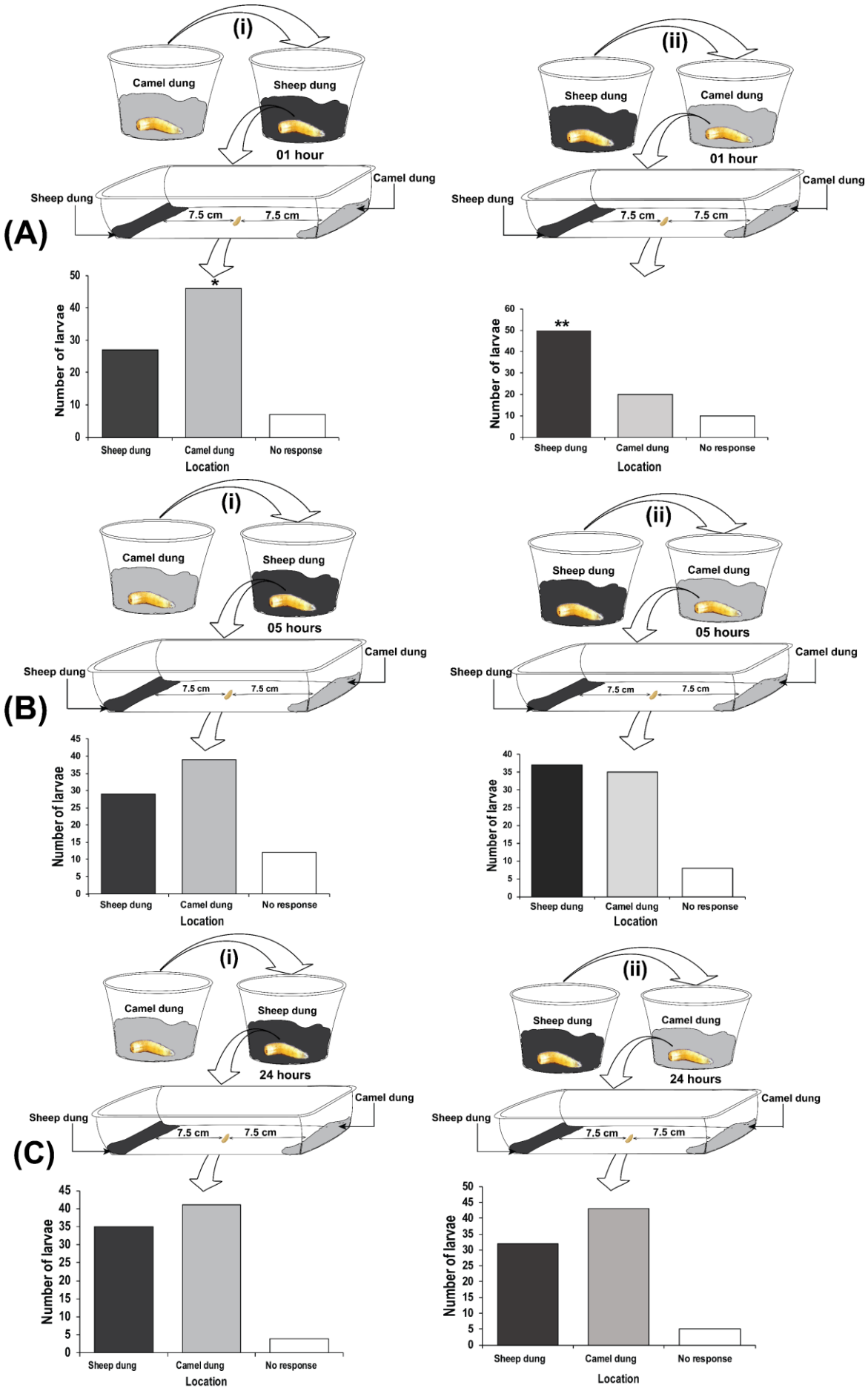
(A)
(i)
Camel dung
Sheep dung
01 hour
Sheep dung
Camel dung
7.5 cm
7.5 cm
*
Number of larvae
Sheep dung
Camel dung
No response
Location
(ii)
Sheep dung
Camel dung
01 hour
Camel dung
Sheep dung
7.5 cm
7.5 cm
**
Number of larvae
Sheep dung
Camel dung
No response
Location
(B)
(i)
Camel dung
Sheep dung
05 hours
Sheep dung
Camel dung
7.5 cm
7.5 cm
Number of larvae
Sheep dung
Camel dung
No response
Location
(ii)
Sheep dung
Camel dung
05 hours
Camel dung
Sheep dung
7.5 cm
7.5 cm
Number of larvae
Sheep dung
Camel dung
No response
Location
(C)
(i)
Camel dung
Sheep dung
24 hours
Sheep dung
Camel dung
7.5 cm
7.5 cm
Number of larvae
Sheep dung
Camel dung
No response
Location
(ii)
Sheep dung
Camel dung
24 hours
Camel dung
Sheep dung
7.5 cm
7.5 cm
Number of larvae
Sheep dung
Camel dung
No response
Location

Figure 5.2. *Stomoxys calcitrans* larval preference for their rearing substrate is retained for a short period (1 hour). (A) Experience test with larvae sojourning in a novel substrate for 1 hour: bioassay with L3 larvae reared on camel dung (i), bioassay with L3 larvae reared on sheep dung (ii). (B) Experience test with larvae sojourning in a novel substrate for 5 hr: bioassay with L3 larvae reared on camel dung (i), bioassay with L3 larvae reared on sheep dung (ii). (C) Experience test with larvae sojourning in a novel substrate for 24 hr: bioassay with L3 larvae reared on camel dung (i), bioassay with L3 larvae reared on sheep dung (ii). Asterisks indicate proportions that differed significantly from random (50%) determined by a two-tailed binomial test: * $p<0.05$, ** $p<0.01$. The absence of asterisks indicates no significant preference by larvae for the substrates provided (two-tailed binomial test: $p >0.05$).

Experience recovery test

In the experience recovery test, I found that, *S. calcitrans* larvae, after being maintained in a novel substrate for 24 hr and transferred back to their original rearing substrate for 24 hours, did not recover a preference for their substrate of provenance. Larvae reared on camel dung, transferred to sheep dung, then put back on camel dung did not significantly prefer camel as predicted (Fig. 5.3A; binomial calculation: 52.1% success, 45.9% failure, $p= 0.741$). Also, larvae reared on sheep dung, transferred to camel dung and returned to sheep dung did not prefer their original rearing substrate (Fig. 5.3B; binomial calculation: 45.3% success, 54.7% failure; $p= 0.32$

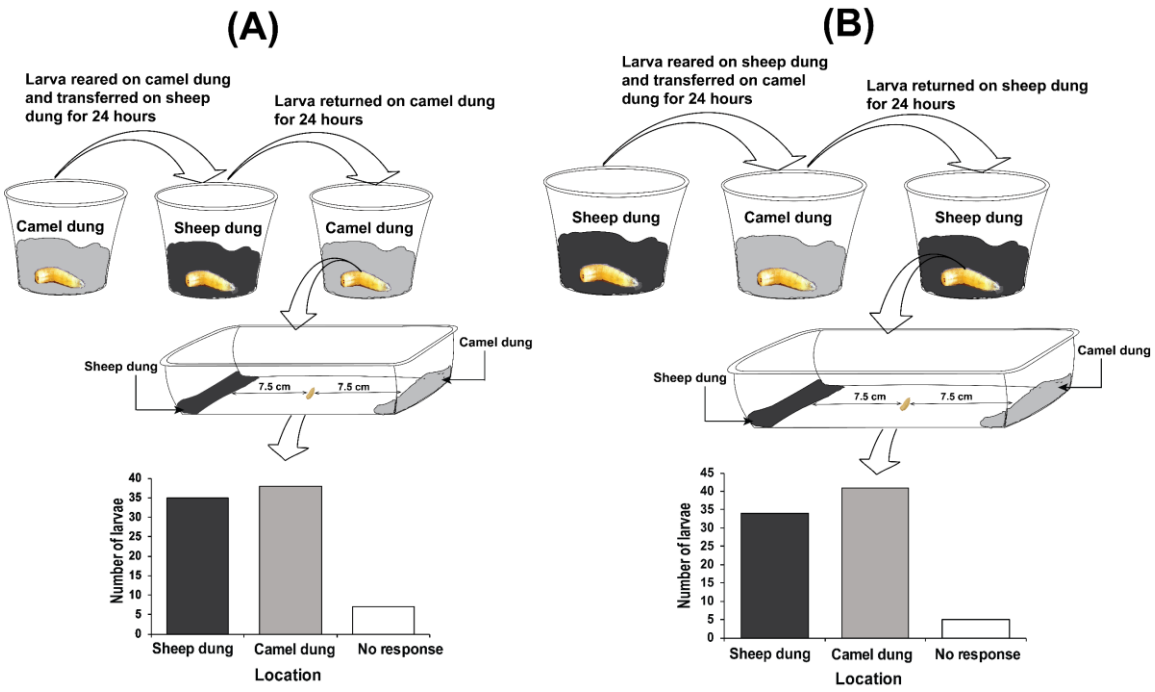

Figure 5.3. *Stomoxys calcitrans* larval preference is not recoverable. (A) Memory recovery test with larvae reared on camel dung. (B) Memory recovery test with larvae reared on sheep dung. In the two tests, there was no significant preference by larvae for the substrates provided (two-tailed binomial test: $p > 0.05$).

Hopkins' host selection principle and chemical legacy hypothesis tests

HHSP Test

According to the result of our oviposition bioassays, *S. calcitrans* gravid females reared on sheep and camel dung did not lay more eggs on sheep and camel dung, respectively, as predicted by the HHSP. The median number of eggs laid on sheep dung and donkey dung by gravid females that emerged from cleaned pupae were not significantly different (Fig. 5.4C.i: U= 49, d.f=1. p=0.97). Also, gravid females from uncleaned pupae laid the same median number of eggs on sheep dung and donkey dung (Fig. 5.4C.ii: U= 45.5, d.f.=1, p=0.762). In the bioassays with females reared on camel dung, gravid females from clean pupae laid more eggs on sheep dung than camel dung (Fig. 5.4D.i: U= 15.5, d.f=1. p=0.01). Similarly, gravid females from unclean pupae laid more eggs on sheep dung than camel dung (Fig. 5.4D.ii: U= 9, d.f=1. p=0.002).

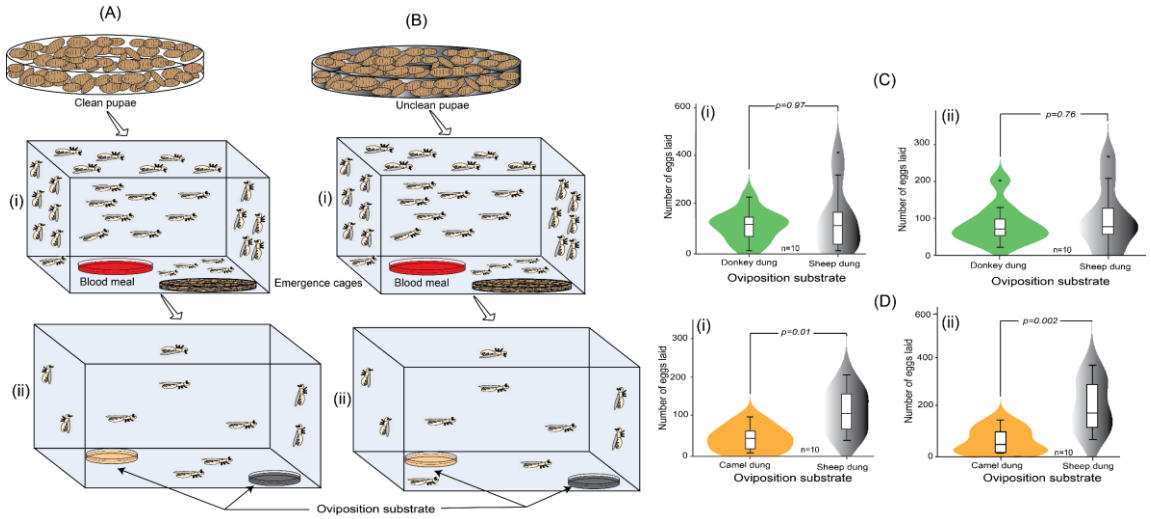

Figure 5.4. Oviposition behaviour in gravid female *S. calcitrans* is not affected by the larvae experience. (A) Oviposition bioassay using *S. calcitrans* gravid females from cleaned pupae: cleaned pupae, adult emergence cage (i), oviposition test arena (ii). (B) Oviposition bioassay using *S. calcitrans* gravid females from uncleaned pupae: uncleaned pupae, adult emergence cage (i), oviposition test arena (ii). (C) Violin plots showing no significant difference (Mann-Whitney test; $p > 0.05$) in number of eggs laid on donkey and sheep dung by gravid females reared on sheep dung when emerging from cleaned pupae (i) and uncleaned pupae (ii). (D) Violin plots showing significant difference (Mann-Whitney test; $p < 0.05$) in number of eggs laid on camel and sheep dung by gravid females reared on camel dung when emerging from clean pupae (i) and unclean pupae (ii). The horizontal bar in each box of the violin plot indicates the median, the extremities of the box indicate the 25-75th percentiles, and whiskers indicate 1.5× the interquartile range from the 25th–75th percentiles; the shape of the violin plot shows the density estimate and extends to extreme values.

Chemical legacy hypothesis test

The chemical legacy hypothesis proposes that chemical cues coating pupae can influence adult oviposition substrate selection. Results of our two oviposition bioassays indicated that gravid females from non-contaminated and contaminated pupae did not exhibit different oviposition behaviour. Gravid females emerged from uncontaminated pupae (Fig 5.5 A.iii: U= 40, d.f=1. p=0.47) and pupae contaminated with sheep dung (Fig 5.5 A.iii: U= 48.5, d.f=1. p=0.94) laid the same number of eggs on rabbit and sheep dung.

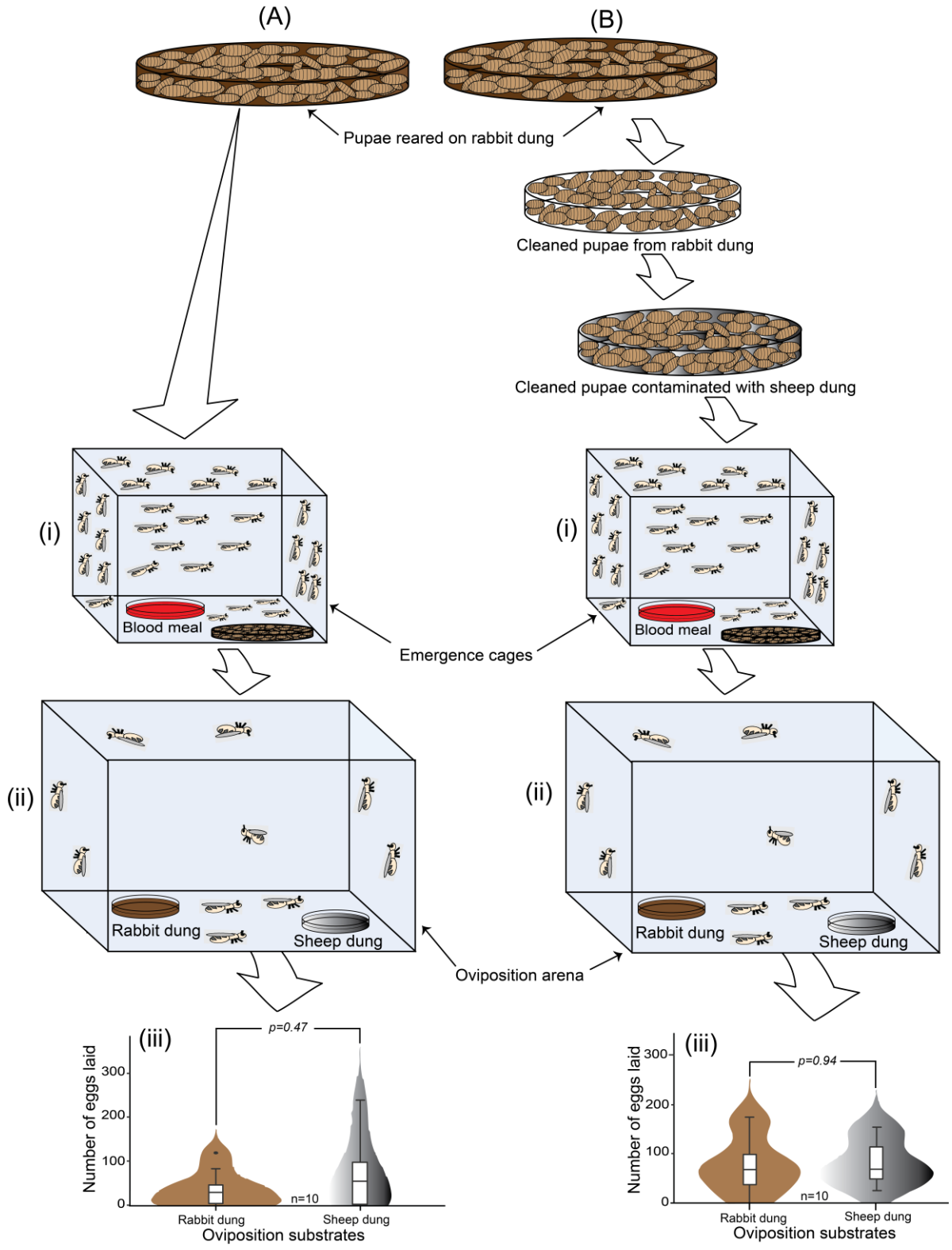
(A)
(B)
Pupae reared on rabbit dung
Cleaned pupae from rabbit dung
Cleaned pupae contaminated with sheep dung
(i)
Blood meal
Emergence cages
(ii)
Rabbit dung
Sheep dung
Oviposition arena
(iii)
p=0.47
p=0.94
Number of eggs laid
300
200
100
0
n=10
Rabbit dung
Sheep dung
Oviposition substrates

Fig. 5.5. Oviposition decision in gravid females of *S. calcitrans* is not affected by the larval chemosensory environment. (a) Oviposition bioassay using S. calcitrans gravid females from non-contaminated pupae: (i) Emergence cage, (ii) oviposition arena, (iii) Violin plots showing no significant difference (Mann–Whitney test; $p > 0.05$) in number of eggs laid on rabbit and sheep dung by gravid females emerged from non-contaminated pupae. (b) Oviposition bioassay using *S. calcitrans* gravid females from pupae contaminated with sheep dung: (i) Emergence cage, (ii) oviposition arena, (iii) Violin plots showing no significant difference (Mann–Whitney test; $p > 0.05$) in number of eggs laid on rabbit and sheep dung by gravid females emerged from pupae contaminated with sheep dung. Bar into each box show the median and those at the extremity of the box show the 25–75th percentiles, which are extended by whiskers indicating 1.5× the interquartile range from the 25th–75th percentiles; the shape denotes the density estimate and extends to extreme values.

5.5 DISCUSSION

I demonstrated that *S. calcitrans* larval preference for rearing substrate was not transferred to the adult stage during metamorphosis. When a rearing substrate was offered singly or paired with another substrate, L3 larvae of *S. calcitrans* preferred the substrate in which they had developed. That preference was disrupted when larvae were exposed to a substrate for 5 hr. The ability of larvae to distinguish their rearing substrate from other substrates has also been observed in other holometabolous insects. Durisko and Dukas (2013) found that *D. melanogaster* larvae reared in a group are attracted by substrates that are occupied or that have been used by other larvae; the same larvae exhibit a significant aversion to fresh food. Similarly, larvae of *Ascia monuste orseis* reared on kale or cabbage prefer the same plants during their fourth and fifth instars when offered a choice including different plants (Santana and Zucoloto 2011). In general, insects staying on a substrate for a period of time develop a preference for that substrate due to the adaptation of their olfactory sensory neurons (Iyengar et al. 2010). The aptitude to recognise their rearing substrate may prevent larvae of *S. calcitrans* from the deleterious effect of leaving a substrate. This was found in *Schistocerca americana*, where leaving one food source in search of another decreases food consumption, increases development time, suppresses growth rate, reduces larval weight, and elevates mortality (Bernays and Bright 2001).

I found that *S. calcitrans* larvae transferred to a new substrate for 5 and 24 hr tend to become adapted to this substrate and lose their preference for the original rearing substrate, even after

having been returned to it. Neuser et al. (2005) explained that experience duration in insects varies from minutes to months, according to the species, age, and gender of the test organism, the strength of the rewarding or aversive stimulus, number of training repetitions, and type of bioassay. Dekker et al. (2006), Linz et al. (2013), Crowley-Gall et al. (2016) all suggest that host shifts in insects presumably alter their olfactory system and consequently modifies their behaviour.

Larval experience did not affect adult oviposition preference in *S. calcitrans*. Gravid females of *S. calcitrans* developed on camel and sheep dung, and emerged from clean pupae, did not prefer to oviposit on their rearing substrate as hypothesized by the HHSP. This was also observed in gravid females that emerged from unwashed pupae, which contradicts the chemical legacy hypothesis. Consequently, our study found no evidence in support of the HHSP. Other studies have also failed to find an effect of larval experience on adult oviposition site preference. Rojas and Wyatt (1999) demonstrated that experience of the cabbage moth caterpillar *Mamestra brassicae* (Lepidoptera: Noctuidae) did not affect oviposition behaviour of their females. Oviposition preference in the milfoil weevil, *Euhrychiopsis lecontei* (Coleoptera: Curculionidae) is not an attribute fixed by larval experience (Solarz and Newman 2001). Kerpel and Moreira (2005) did not find Hopkins′ effect in *Heliconius erato* (Lepidoptera: Nymphalidae). In *Polygonia c-album* (Lepidoptera: Nymphalidae) larval diet also had no effect on egg-laying decisions of the adult female (Janz et al. 2009).

A possible explanation for a lack of connection between the larval rearing substrate and adult female oviposition preference is offered by the neo-Hopkins principle developed by Jaenike (1983), where host preference is determined by experience gained by the adult after emergence. The flight capability of *S. calcitrans* adult is relatively high [5 km; see Taylor et al. (2010)]. During the flying period, *S. calcitrans* adult experience different kinds of visual and chemical cues that may shape adult behavioural choice. An alternative to this explanation may be the remodelling of the mushroom body, which is a structure in the insect brain essential for olfactory learning and memory. For instance, in *Drosophila*, olfactory neurons forming the mushroom body are subject to dramatic changes in axonal projection pattern and dendrite and axon morphology during metamorphosis, which may affect transfer of information to the adult stage (Heisenberg 1998; Lee et al. 1999; Zwarts et al. 2015). Barron (2001) also implicates the influence of genetic plasticity in insect host preference.

Stomoxys calcitrans is a cosmopolitan species (Dsouli-Aymes et al. 2011); among the 18 species that encompass the *Stomoxys* genus, only *S. calcitrans* has a worldwide distribution (Zumpt 1973). Larvae of *S. calcitrans* are polyphagous; they feed and develop on a wide range of substrates including dung (including camel, cow, horse, donkey and sheep), and decaying plant materials (such as hay, alfalfa, silage, sugarcane and compost). Compared to specialist insects, the olfactory system of generalist insects is less sensitive (Zhang et al. 2013; Conchou et al. 2017), and according to the "neural constraints hypothesis", generalist insects make less accurate decisions when selecting substrates (Sadtler et al. 2014; Wang 2017). This renders their behaviour difficult to predict.

5.6 CONCLUSION

Overall, the current study has demonstrated that *S. calcitrans* larval preference is not transferred to the adult during metamorphosis. *Stomoxys calcitrans* gravid females did not prefer to lay eggs on the substrate in which they developed. Consequently, our findings do not match the expectations of the Hopkins's host selection hypothesis. I suggest that there is a need for additional research on the applicability of the HHSP in generalist insects characterised by a high capacity for adaptation and flexibility in use of substrates for development.

CHAPTER 6 General discussion

This thesis examined oviposition decisions by gravid female *Stomoxys calcitrans*, the associated fitness consequences, the olfactory cue(s) implicated, and the influence of biotic interactions and larval experience on these decisions.

In Chapter 2, I investigated the preference of gravid female *S. calcitrans* for various potential oviposition substrates and I also asked why this decision is made. I found that gravid female *S. calcitrans* laid more eggs on donkey followed by sheep dung, corroborating the observations of Hafez and Gamal-Eddin (1959). I demonstrated that this preference was related to the performance of *S. calcitrans* offspring on these substrates with high nutritional value. This confirmed the preference-performance hypothesis, which proposes that gravid females of holometabolous insects prefer to oviposit on substrates that will ensure the fitness of the pre-imaginal stages of their offspring (Jaenike 1978). In addition, I showed that this preference-performance behaviour in gravid female *S. calcitrans* was mediated by β-citronellene and carvone, two volatile chemicals isolated from donkey and sheep dung, respectively. These two compounds elicited the strongest egg-deposition under laboratory conditions and enhanced *S. calcitrans* catches in a field trial, showing the preferred substrate can be represented using single semiochemicals. This chapter highlights the potential use of β-citronellene and carvone in the surveillance and management of *S. calcitrans*.

Because Chapter 2 only assessed the effect of abiotic factors (olfactory cues, physical and chemical constituents of the substrates) on *S. calcitrans* oviposition behaviour, I aimed in Chapter 3 to elucidate the influence of biotic factors (competition and parasitism) on *S. calcitrans* oviposition decisions. I demonstrated that either in the light or dark, gravid female *S. calcitrans* avoided substrates with their conspecific larvae and larvae of the house fly, *Musca domestica.* This suggests that avoidance behaviour is associated with olfactory cues. Similarly, Seenivasagan et al (2009) showed that the alkane n-heneicosane emitted by *Aedes aegypti* larvae repels gravid females of this mosquito. The same alkane also deters oviposition in *Aedes albopictus* (Gonzalez et al. 2014). Additionally, I found that gravid female *S. calcitrans* failed to distinguish between substrates with and without the mite *Macrocheles muscaedomesticae* in darkness but avoided mite-infested substrates with mites under light. This indicated that female *S. calcitrans* use visual cues to avoid parasite-infested substrates. In *Drosophila melanogaster*, Kacsoh et al (2013) demonstrated that gravid females used visual cues to discriminate between substrates infested or free of parasites. Furthermore, in this chapter, I demonstrated that gravid female *S. calcitrans* avoid substrates with con- and heterospecific larvae and mites because of the intra- and interspecific

competition and parasitism that their offspring could face during pre-imaginal stages. This result supported the preference-performance oviposition behaviour demonstrated by *S. calcitrans* in Chapter 2.

To continue assessing the fitness consequences related to *S. calcitrans* oviposition decisions, I aimed in Chapter 4 to study the effect of larval densities and substrate quality on *S. calcitrans* wing morphology. I found that flies developing in a group of 5 had larger wing centroid size, wing length, wing width, wing area and wing loading compared with those reared in a group of 25. Also, flies developed in donkey and sheep dung (preferred oviposition substrates; Chapter 2) had larger wing centroid size, wing length, wing width, wing area and wing loading in comparison with those developing in camel and cow dung. Furthermore, the wing shape of *S. calcitrans* changed significantly across these substrates and larval densities. These results were consistent with studies on *Drosophila* (Soto et al. 2008), and *Ae. aegypti* and *Ae. albopictus* (Stephens and Juliano 2012), which reported the influence of developmental substrate and larval densities on the wing geometry of these insects. Also, the results of this chapter provide an additional explanation for why *S. calcitrans* preferred to lay on donkey and sheep dung (Chapter 2), or on a substrate with lower larval density (Chapter 3). Given that long and large wings positively affect the flight performance of insects (Kay and Muir 1998; Berwaerts et al. 2002; Davis and Holden 2015), these results suggest that gravid females lay more eggs on donkey and sheep dung or on substrates with lower larval density because these substrates will increase the flight efficacy of their offspring.

The Hopkins' host selection principle (HHSP) proposes that, in holometabolous insects, gravid females prefer to lay on substrates in which they developed as larvae. To assess the possible implication of this hypothesis in my model insect, I aimed in Chapter 5 to determine the effect of larval experience on *S. calcitrans* oviposition decisions. I found that *S. calcitrans* larvae reared on camel or sheep dung were able to recognise the substrate in which they developed. This confirmed the observations of Santana and Zucoloto (2011) and Durisko and Dukas (2013) in *Ascia monuste orseis* and *D. melanogaster*, respectively. Bernays (1995) and Iyengar et al. (2010) explained that when an insect comes into contact with and feeds on a substrate, it develops a preference for that substrate due to adaptation of their olfactory sensory neurons. When I tested whether this aptitude of *S. calcitrans* larvae to recognise their rearing substrate persisted through metamorphosis into the adult stage, I found that gravid females did not prefer to oviposit on the substrate in which they developed. Therefore, I ruled out the possible influence of larval chemosensory environment on

oviposition decisions by *S. calcitrans*. This finding corroborated results of other studies (Rojas and Wyatt 1999; Solarz and Newman 2001; Kerpel and Moreira 2005; Janz et al. 2009), which found no persistence of larval experience into the adult stage. This chapter supports the neo-Hopkins hypothesis (Jaenike 1983), suggesting that in insects, adult behaviour is shaped by the olfactory and visual cues encountered after emergence. Alternatively, these results could also suggest that the choice of oviposition sites is innate in the *S. calcitrans*.

6.1 IMPLICATIONS

Overall, my thesis provides detailed information of how gravid female *S. calcitrans* cope with biotic (intra- and interspecific competition, parasitism, larval experience, etc…) and abiotic (olfactory cues, visuals cues, substrate physicochemical properties, etc…) factors when selecting oviposition substrates.

I have shown that donkey and sheep dung is preferred by gravid female *S. calcitrans* for oviposition. I predict that in areas where donkey and sheep are major livestock, population density of *S. calcitrans* may be higher owing to the presence of suitable oviposition sites for gravid females. It has already been revealed that the increase of *S. calcitrans* population is related to the presence of suitable breeding sites. For instance, Cook et al (2018) demonstrated that *S. calcitrans* developed in higher numbers from vegetable crop residues such as celery, leek, broccoli, cabbage, lettuce and silverbeet. Cançado et al (2013) in Brazil and Solórzano et al (2015) in Costa Rica found that outbreaks of *S. calcitrans* are associated with an increase in sugar cane and pineapple production. The two oviposition attractants (β-citronellene and carvone) that I have identified could play an important role in reducing the abundance of *S. calcitrans* by luring and killing gravid females. Placement of traps baited with β-citronellene and carvone around places where donkeys and sheep are bred, the intensity of *S. calcitrans* infestation could be significantly reduced by reducing the population of *S. calcitrans* gravid females and thereby suppressing population growth.

In this thesis, I also demonstrated that the mite *M. muscaedomesticae* considerably reduced *S. calcitrans* eggs hatchability, and larval and adult survival. Under semi-field conditions, Rodriguez *et al*. (1970) demonstrated that this mite can reduce house fly, *Musca domestica*, abundance by 86-99%. Therefore, there is the potential to use these mites as a form of biological control for *S. calcitrans*. If mass produced and spread on *S. calcitrans* oviposition

substrates, augmentative biological control using *M. muscaedomesticae* could minimise the abundance of *S. calcitrans* and lead to reduced blood-feeding and disease transmission.

6.2 FUTURE DIRECTIONS

My thesis has generated important knowledge about the oviposition behaviour of *Stomoxys calcitrans*. It has also opened a starting point for several research projects among others: (1) the identification of chemical cues triggering the avoidance of substrate with conspecific and heterospecific larvae in *S. calcitrans* gravid females, (2) optimisation of the discovered attractants (β-citronellene and carvone) and repellents (Once identified), (3) development of a push-pull control system against *S. calcitrans* based on the identified attractants and repellents, and (4) elucidation of the *S. calcitrans* olfactory neurons implicated in the detection of the identified attractants and repellents.

A chemical that prevents an insect from reaching a valued resource (breeding site, food, sexual partner, etc...) is called repellent (Barton-Browne 1977). In insect pest management, application of repellent molecules onto the skin of a host or volatilised can protect them from insect bites (Achee et al. 2012). In my case, it would be useful to identify molecules emitted by conspecific and heterospecific larvae, that prevent *S. calcitrans* gravid females from laying eggs. However, it is important to mention that knowing the identity of a candidate attractant or repellent is not enough; the concentration in which the molecule is effective is also required (Reisenman et al. 2016). To obtain this information, optimisation studies where the identified candidate attractants (or repellents) are tested at various concentrations are needed, while making use of different dispenser and trap devices, at different positions, localities and seasons. This is to ensure that the molecule elicits the strongest behavioural activity (attractiveness or repellency).

Once the efficacy of the attractant and repellent is tested and confirmed, it would be worthwhile to associate them in a system called "*Push-Pull Technology*" (PPT). In this system, the repellent prevents the harmful insects (e.g. *S. calcitrans*) from reaching the host by causing movement of the insect away from the protected host (push). The associated attractant will be used simultaneously to trap the harmful insects (pull) (Hassanali, et al. 2008). This technology has already been effective in reducing vector-host interactions in various systems (e.g. *Anopheles gambiae* (Menger et al. 2014) *Aedes aegypti* (Obermayr et al. 2015), *Anopheles albimanus*, *Anopheles vestitipennis* (Wagman et al. 2015)).

Lastly, it is also important to examine processes by which the discovered attractants and repellents are perceived by the *S. calcitrans* neural system (from the peripheral sensory organ (sensilla) to the primary olfactory centre (antennal lobe) and then to the higher brain (mushroom body and lateral horn)). Delineating this olfactory circuit will help to determine how odours are detected, encoded into neural activity and translated to a behavioural output (attraction or repulsion) in *S. calcitrans*.

6.3 CLOSING REMARKS

The main objective of my thesis was to study the oviposition decisions in *Stomoxys calcitrans*, the associated fitness consequences for offspring, the olfactory cue(s) involved, and the influence of biotic factors in egg-laying decisions. My thesis clearly demonstrates the existence of olfactory-guided preference-performance oviposition behaviour in *S. calcitrans* gravid females. I have identified two odourant molecules (β-citronellene and carvone) used by the females of this fly to locate substrates with good nutritional value for their progeny. Most interestingly, I showed that when those molecules are incorporated in a field trapping system, they significantly enhance *S. calcitrans* catches. Furthermore, without identifying the olfactory cues implicated, I demonstrated that *S. calcitrans* gravid females use their sense of smell to avoid substrates with competitors. This suggests that further studies aiming to elucidate the molecules responsible for this repellency are needed.

CHAPTER 7 References

Abràmoff DMD (2004) Image processing with ImageJ. Biphotonics International 7

Achee NL, Bangs MJ, Farlow R, Killeen GF, Lindsay S, Logan JG, et al. (2012) Spatial repellents: from discovery and development to evidence-based validation. Malaria Journal. 11: 164.doi:10.1186/1475-2875-11-164

Afify A, Galizia CG (2015) Chemosensory cues for mosquito oviposition site selection. Journal of Medical Entomology 52:120–130. doi: 10.1093/jme/tju024

Ahmad M, Chaudhary SU, Afzal AJ, Tariq M (2015) Starvation-induced dietary behaviour in *Drosophila melanogaster* larvae and adults. Scientific Reports 5: doi: 10.1038/srep14285

Ali SAI, Diakite MM, Ali S, Wang M-Q (2015) Understanding insect behaviors and olfactory signal transduction. Journal of Genetics, Molecular and Cellular Biology2:10

Alto BW, Lounibos LP, Higgs S, Juliano SA (2005) Larval competition differentially affects arbovirus infection in *Aedes* mosquitoes. Ecology 86:3279–3288. doi: 10.1890/05-0209

Alves VM, Moura MO, de Carvalho CJB (2016) Wing shape is influenced by environmental variability in *Polietina orbitalis* (Stein) (Diptera: Muscidae). Revista Brasileira de Entomologia 60:150–156. doi: 10.1016/j.rbe.2016.02.003

Arn H, Städler E, Rauscher S (1975) The electroantennographic detector — A selective and sensitive tool in the gas chromatographic analysis of insect pheromones. Zeitschrift für Naturforschung C 30:722–725. doi: 10.1515/znc-1975-11-1204

Axtell RC (1963) Acarina occurring in domestic animal manure 1. Annals of the Entomological Society of America 56:628–633. doi: 10.1093/aesa/56.5.628

Averill AL, Prokopy RJ (1987) Intraspecific competition in the Tephritid fruit fly *Rhagoletis Pomonella*. Ecology 68:878–886. doi: 10.2307/1938359

Baglan H, Lazzari C, Guerrieri F (2017) Learning in mosquito larvae (*Aedes aegypti*): Habituation to a visual danger signal. Journal of Insect Physiology 98:160–166. doi: 10.1016/j.jinsphys.2017.01.001

Baldacchino F, Muenworn V, Desquesnes M, et al (2013) Transmission of pathogens by *Stomoxys* flies (Diptera, Muscidae): a review. Parasite 20:26. doi: 10.1051/parasite/2013026

Baleba SBS, Torto B, Masiga D, et al (2019) Egg-laying decisions based on olfactory cues enhance offspring fitness in *Stomoxys calcitrans* L. (Diptera: Muscidae). Scientific Reports 9: 3850. doi: 10.1038/s41598-019-40479-9

Barragan-Fonseca KB, Dicke M, van Loon JJA (2018) Influence of larval density and dietary nutrient concentration on performance, body protein, and fat contents of black soldier fly larvae (*Hermetia illucens*). Entomologia Experimentalis et Applicata 166:761–770. doi: 10.1111/eea.12716

Barron AB (2001) The Life and Death of Hopkins' Host-Selection Principle. Journal of Insect Behavior 13

Barton-browne L (1977)."Host-related responses and their suppression: some behavioral considerations,"*in* Chemical control of insect behavior: theory and application, eds Shorey HH and McKelvey JJ. (New York, NY: Wiley), 117–127.

Benelli G (2014) Aggressive behavior and territoriality in the olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae): Role of residence and time of day. Journal of Insect Behavior 27:145–161. doi: 10.1007/s10905-013-9411-7

Benelli G, Donati E, Romano D, et al (2016) Is bigger better? Male body size affects wing-borne courtship signals and mating success in the olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae): Size and mating success in B. oleae. Insect Science 23:869–880. doi: 10.1111/1744-7917.12253

Bengtsson JM, Wolde-Hawariat Y, Khbaish H, Negash M, Jembere B, Seyoum E, Hillbur Y (2009) Field attractants for *Pachnoda interrupta* selected by means of GC-EAD and single sensillum screening. Journal of Chemical Ecology 35: 1063–1076.

Bernays E., Bright K. (2001) Food choice causes interrupted feeding in the generalist grasshopper Schistocerca americana: further evidence for inefficient decision-making. Journal of Insect Physiology 47:63–71. doi: 10.1016/S0022-1910(00)00090-1

Bentley MD, Day JF (1989) Chemical ecology and behavioral aspects of mosquito oviposition. Annual review of entomology 34:401–421

Berwaerts K, Dyck HV, Aerts P (2002) Does flight morphology relate to flight performance? An experimental test with the butterfly *Pararge aegeria*. Functional Ecology 16:484–491. doi: 10.1046/j.1365-2435.2002.00650.

Betts CR, Wootton RJ (1988) Wing shape and flight behaviour in butterflies (lepidoptera: papilionoidea and hesperioidea): a preliminary analysis. Journal of Experimental Biology 138: 271:288

Bhattacharya A, Kaliwal BB (2005) The biochemical effects of potassium chloride on the silkworm, (Bombyx mori L.). Insect Science 12:95–100. doi: 10.1111/j.1744-7917.2005.00011.

Bidlingmayer WL (1994) How mosquitoes see traps: role of visual responses. Journal of the American Mosquito Control Association, 10: 272-27

Birkett MA, Agelopoulos N, Jensen KMV (2004) The role of volatile semiochemicals in mediating host location and selection by nuisance and disease-transmitting cattle flies. Medical and Veterinary Entomology 18: 313–322.

Bisch-Knaden S, Dahake A, Sachse S, et al (2018) Spatial representation of feeding and oviposition odors in the brain of a hawkmoth. Cell Reports 22:2482–2492. doi:

Blackiston DJ, Silva Casey E, Weiss MR (2008) Retention of memory through metamorphosis: Can a moth remember what it learned as a caterpillar? PLoS ONE 3:e1736. doi: 10.1371/journal.pone.0001736

Boss CB, Fredeen KJ (2004) Concepts, instrumentation and techniques in inductively coupled plasma optical emission spectrometry. 120

Breiman L (2001) Random forests. Machine learning 45:5–32

Bremner JM (1996) Nitrogen total. In Sparks DL, editors. Methods of soil analysis, Part 3 chemical methods. Soil Science Society of America, American Society of Agronomy, Madison, WI,1085–1121.

Bretz F, Hothorn T, Westfall PH (2011) Multiple comparisons using R. CRC Press, Boca Raton, FL

Broce AB, Haas MS (1999) Relation of cattle manure age to colonization by stable fly and house fly (Diptera: Muscidae). Journal of the Kansas Entomological Society 60–72

Burrack HJ, Fornell AM, Connell JH, et al (2009) Intraspecific larval competition in the olive fruit fly (Diptera: Tephritidae). Environmental Entomology 38:1400–1410. doi: 10.1603/022.038.0508

Cançado PH, Ferreira T, Piranda EM, Soares CO (2013) Sugarcane stems as larval habitat for the stable fly (*Stomoxys calcitrans*) in sugarcane plantations. Pesquisa Veterinária Brasileira 33:741–744

Carn VM (1996) The role of dipterous insects in the mechanical transmission of animal viruses. British Veterinary Journal 152: 377–393.

Carvalho CJB de (2005) A catalogue of the Muscidae (Diptera) of the Neotropical Region. Magnolia Press, Auckland, N.Z.

Chang W (2013) R graphics cookbook: practical recipes for visualizing data, first edition. O'Reilly, Beijing Cambridge Farnham Köln Sebastopol Tokyo

Changbunjong T, Sumruayphol S, Weluwanarak T, et al (2016) Landmark and outline-based geometric morphometrics analysis of three *Stomoxys* flies (Diptera: Muscidae). Folia Parasitologica 63: doi: 10.14411/fp.2016.037

Chen Y, Ruberson JR, Olson DM (2008) Nitrogen fertilization rate affects feeding, larval performance, and oviposition preference of the beet armyworm, *Spodoptera exigua*, on cotton. Entomologia Experimentalis et Applicata 126:244–255. doi: 10.1111/j.1570-7458.2007.00662.

Clark KE, Hartley SE, Johnson SN (2011) Does mother know best? The preference-performance hypothesis and parent-offspring conflict in aboveground-belowground herbivore life cycles. Ecological Entomology 36:117–124. doi: 10.1111/j.1365-2311.2010.01248.

Cook DF, Telfer DV, Lindsey JB, Deyl RA (2018) Substrates across horticultural and livestock industries that support the development of stable fly, *Stomoxys calcitrans* (Diptera: Muscidae): Substrates that support stable fly. Austral Entomology 57:344–348. doi: 10.1111/aen.12282

Conchou L, Anderson P, Birgersson G (2017) Host plant species differentiation in a polyphagous moth: Olfaction is enough. Journal of Chemical Ecology 43:794–805. doi: 10.1007/s10886-017-0876-2

Corbet SA (1985) Insect chemosensory responses: a chemical legacy hypothesis. Ecological Entomology 10:143–153. doi: 10.1111/j.1365-2311. 1985.tb00543.x

Couto A, Alenius M, Dickson BJ (2005) Molecular, anatomical, and functional organization of the *Drosophila* olfactory system. Current Biology 15, 1535–1547.

Cilek JE (1999) Evaluation of various substances to increase adult *Stomoxys calcitrans* (Diptera: Muscidae) collections on Alsynite cylinder traps in North Florida. Journal of Medical Entomology. 36: 605-609.

Cilek JE, Greene GL (1994) Stable fly (Diptera: Muscidae) insecticide resistance in Kansas cattle feedlots.Journal of Economic Entomology 87: 275–279

Craig TP, Itami JK, Price PW (1989) A strong relationship between oviposition preference and larval performance in a shoot-alling sawfly. Ecology 70:1691–1699. doi: 10.2307/1938103

Crnjar R, Scalera G, Liscia A, et al (1989) Morphology and EAG mapping of the antennal olfactory receptors in *Dacus oleae*. Entomologia Experimentalis et Applicata 51:77–85. doi: 10.1111/j.1570-7458. 1989.tb01216.

Crowley-Gall A, Date P, Han C, et al (2016) Population differences in olfaction accompany host shift in *Drosophila mojavensis*. Proceedings of the Royal Society B: Biological Sciences 283:20161562. doi: 10.1098/rspb.2016.1562

Dalgaard P (2008) Introductory statistics with R. Springer New York, New York, NY

Dam NMV, Poppy GM (2008) Why plant volatile analysis needs bioinformatics – detecting signal from noise in increasingly complex profiles. Plant Biology 10:29–37. doi: 10.1055/s-2007-964961

Danchin E, Wagner RH (1997) The evolution of coloniality: the emergence of new perspectives. Trends in Ecology & Evolution 12:342–347. doi: 10.1016/S0169-5347(97)01124-5

Darshanee HLC, Ren H, Ahmed N, et al (2017) Volatile-mediated attraction of greenhouse whitefly *Trialeurodes vaporariorum* to tomato and eggplant. Frontiers in Plant Science 8:. doi: 10.3389/fpls.2017.01285

Davis AK, Holden MT (2015) Measuring intraspecific variation in flight-related morphology of monarch butterflies (*Danaus plexippus*): Which sex has the best flying gear? Journal of Insects 2015:1–6. doi: 10.1155/2015/591705

Dawit L, Addis M, Gari G (2012) Distribution, seasonality and relative abundance of *Stomoxys* flies in selected districts of central Ethiopia. World Applied Sciences Journal 19:998–1002

De Bruyne M, Clyne PJ, Carlson JR (1999) Odor coding in a model olfactory organ: The *Drosophila* maxillary palp. Journal of Neuroscience. 19: 4520–4532.

De Mendiburu F (2017) Agricolae: statistical procedures for agricultural research. R packa ge version 1.2-8. https://CRAN.R-project.org/package=agricolae

De Moraes CM, Stanczyk NM, Betz HS, et al (2014) Malaria-induced changes in host odors enhance mosquito attraction. Proceedings of the National Academy of Sciences 111:11079–11084. doi: 10.1073/pnas.1405617111

DeVries PJ, Penz CM, Hill RI (2010) Vertical distribution, flight behaviour and evolution of wing morphology in morpho butterflies: wing evolution in morpho butterflies. Journal of Animal Ecology 79:1077–1085. doi: 10.1111/j.1365-2656.2010. 01710.x

Dekker T, Ibba I, Siju KP, et al (2006) Olfactory shifts parallel superspecialism for toxic Fruit in *Drosophila melanogaster* sibling, *D. sechellia*. Current Biology 16:101–109. doi: 10.1016/j.cub.2005.11.075

Diehl PA, Vlimant M, Guerenstein PG, Guerin PM (2003) Ultrastructure and receptor cell responses of the antennal grooved peg sensilla of *Triatoma infestans* (Hemiptera: Reduviidae). Arthropod Structure & Development 31: 271–285.

Dogan M, Gunay F, Puggioli A, et al (2016) Establishment of a satellite rearing facility to support the release of sterile *Aedes albopictus* males. I. Optimization of mass rearing parameters. Acta Tropica 159:62–68. doi: 10.1016/j.actatropica.2016.03.032

Dsouli-Aymes N, Michaux J, De Stordeur E, et al (2011) Global population structure of the stable fly (*Stomoxys calcitrans*) inferred by mitochondrial and nuclear sequence data. Infection, Genetics and Evolution 11:334–342. doi: 10.1016/j.meegid.2010.11.001

Du Y, Millar JG (1999) Electroantennogram and oviposition bioassay responses of *Culex quinquefasciatus* and *Culex tarsalis* (Diptera: Culicidae) to chemicals in odors from Bermuda grass infusions. Journal of Medical Entomology 36:158–166. doi: 10.1093/jmedent/36.2.158

Dujardin J-P (2008) Morphometrics applied to medical entomology. Infection, Genetics and Evolution 8:875–890. doi: 10.1016/j.meegid.2008.07.011

Durisko Z, Dukas R (2013) Attraction to and learning from social cues in fruitfly larvae. Proceedings of the Royal Society Biological Sciences 280:20131398–20131398. doi: 10.1098/rspb.2013.1398

Dutra HLC, Lopes da Silva V, da Rocha Fernandes M, et al (2016) The influence of larval competition on Brazilian Wolbachia-infected *Aedes aegypti* mosquitoes. Parasites & Vectors 9: doi: 10.1186/s13071-016-1559-5

Duyck P-F, David P, Quilici S (2004) A review of relationships between interspecific competition and invasions in fruit flies (Diptera: Tephritidae). Ecological Entomology 29:511–520. doi: 10.1111/j.0307-6946.2004.00638.

Dweck HKM, Ebrahim SAM, Kromann S, et al (2013) Olfactory preference for egg laying on citrus substrates in *Drosophila*. Current Biology 23:2472–2480. doi: 10.1016/j.cub.2013.10.047

Eberl DF, Hardy RW, Kernan MJ (2000) Genetically similar transduction mechanisms for touch and hearing in *Drosophila*. The Journal of Neuroscience 20:5981–5988.

Ganesan K, Mendki MJ, Suryanarayana MVS, et al (2006) Studies of *Aedes aegypti* (Diptera: Culicidae) ovipositional responses to newly identified semiochemicals from conspecific eggs. Australian Journal of Entomology 45:75–80. doi: 10.1111/j.1440-6055.2006.00513.

Gerson U, Smiley RL, Ochoa R, Gerson U (2003) Mites (acari) for pest control, 2nd ed. Blackwell Science, Oxford; Malden, MA

Gilles J, David J-F, Duvallet G (2005) Temperature effects on development and survival of two stable flies *Stomoxys calcitrans* and *Stomoxys niger niger* (Diptera: Muscidae), in La Réunion Island. Journal of Medical Entomology 42: 260–265. doi: 10.1603/0022-2585(2005)042[0260: TEODAS]2.0.CO;2

Ferrari MCO, Messier F, Chivers DP (2008) Threat-sensitive learning of predators by larval mosquitoes *Culex restuans*. Behavioral Ecology and Sociobiology 62:1079–1083. doi: 10.1007/s00265-007-0535-7

Fleischer J, Pregitzer P, Breer H, Krieger J (2018) Access to the odor world: olfactory receptors and their role for signal transduction in insects. Cellular and Molecular LifeSciences 75: 485-508. doi: 10.1007/s00018-017-2627-5

Foil LD, Hogsette JA (1994) Biology and control of tabanids, stable flies and horn flies. Revue scientifique et technique de l'Office international des epizooties 13: 1125–1158.

Fraimout A, Jacquemart P, Villarroel B, et al (2018) Phenotypic plasticity of *Drosophila suzukii* wing to developmental temperature: implications for flight. The Journal of Experimental Biology 221: jeb166868. doi: 10.1242/jeb.166868

Frago E, Bauce É (2014) Life-history consequences of chronic nutritional stress in an outbreaking insect defoliator. PloS one 9: e88039

Friesen K, Berkebile DR, Zhu JJ, Taylor DB (2017) Augmenting laboratory rearing of stable fly (Diptera: Muscidae) larvae with ammoniacal salts. Journal of Insect Science 17:21. doi: 10.1093/jisesa/iew119

Gandolfi M, Mattiacci L, Dorn S (2003) Preimaginal learning determines adult response to chemical stimuli in a parasitic wasp. Proceedings of the Royal Society B: Biological Sciences 270:2623–2629. doi: 10.1098/rspb.2003.2541

Getahun MN, Thoma M, Lavista-Llanos S, et al (2016) Intracellular regulation of the insect chemoreceptor complex impacts odour localization in flying insects. The Journal of Experimental Biology 219:3428–3438. doi: 10.1242/jeb.143396

Ghaninia M, Larsson M, Hansson BS, Ignell R (2008) Natural odor ligands for olfactory receptor neurons of the female mosquito *Aedes aegypti*: use of gas chromatography-linked single sensillum recordings. Journal of Experimental Biology 211:3020–3027. doi: 10.1242/jeb.016360

Ghaninia M, Hansson BS, Ignell R (2007). The antennal lobe of the African malaria

mosquito, *Anopheles gambiae*-innervation and three-dimensional reconstruction. Arthropod Structure & Development 36: 23–39.

Giunti G, Palmeri V, Algeri GM, Campolo O (2018) VOC emissions influence intra- and interspecific interactions among stored-product Coleoptera in paddy rice. Scientific Reports 8: doi: 10.1038/s41598-018-20420-2

Gómez Jiménez MI, Sarmiento CE, Díaz MF, et al (2014) Oviposition, larval preference, and larval performance in two polyphagous species: does the larva know best? Entomologia Experimentalis et Applicata 153:24–33. doi: 10.1111/eea.12225

Gonzalez PV, Audino PAG, Masuh HM (2014) Electrophysiological and behavioural response of *Aedes albopictus* to n-heinecosane, an ovipositional pheromone of *Aedes aegypti*. Entomologia Experimentalis et Applicata 151:191–197. doi: 10.1111/eea.12184

Gonzalez PV, González Audino PA, Masuh HM (2016) Oviposition behavior in *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in response to the presence of heterospecific and conspecific larvae. Journal of Medical Entomology 53:268–272. doi: 10.1093/jme/tjv189

Gómez GF, Márquez EJ, Gutiérrez LA, et al (2014) Geometric morphometric analysis of Colombian *Anopheles albimanus* (Diptera: Culicidae) reveals significant effect of environmental factors on wing traits and presence of a metapopulation. Acta Tropica 135:75–85. doi: 10.1016/j.actatropica.2014.03.020

González-Santoyo I, Córdoba-Aguilar A (2012) Phenoloxidase: a key component of the insect immune system. Entomologia Experimentalis et Applicata 142:1–16. doi: 10.1111/j.1570-7458.2011.01187.

Grabe V, Sachse S (2018) Fundamental principles of the olfactory code. BioSystems 164: 94-101.

Greene GL, Hogsette JA, Patterson RS (1989) Parasites that attack stable flyand house fly (Diptera: Muscidae) puparia durin the winter on diaries in northerwestern Florida. Journal of Economical Entomology 82: 412-415.

Guerenstein, PG, Christensen, TA, Hildebrand, JG (2004). Sensory processing of ambient CO information in the brain of the moth *Manduca sexta*. Journal of Comparative Physiology 190: 707–725.

Guglielmone AA, Volpogni MM, Quaino OR, et al (2004) Abundance of stable flies on heifers treated for control of horn flies with organophosphate impregnated ear tags. Medical and Veterinary Entomology 18:10–13. doi: 10.1111/j.0269-283x.2004.0466.

Guidobaldi F, Guerenstein PG (2015) Oviposition in the blood-sucking insect *Rhodnius prolixus* is modulated by host odors. Parasites & Vectors 8: doi: 10.1186/s13071-015-0867-5

Guidobaldi F, May-Concha IJ, Guerenstein PG (2014) Morphology and physiology of the olfactory system of blood-feeding insects. Journal of Physiology-Paris 108:96–111. doi: 10.1016/j.jphysparis.2014.04.006

Gutierrez C, Corbera JA, Juste MC, et al (2005) An outbreak of abortions and high neonatal mortality associated with *Trypanosoma evansi* infection in dromedary camels in the Canary Islands. Veterinary Parasitology 130:163–168. doi: 10.1016/j.vetpar.2005.02.009

Hafez M, Gamal-Eddin FM (1959) Ecological studies on *Stomoxys calcitrans* L. and *Sitiens* Rond,in Egypt, with suggestions on their control. *Bulletin de la Société Entomologique d 'Egypte* XLIII 245 –283.

Hammer Ø, Harper, David AT, Ryan PD (2001) Past: Paleontological Statistics Software package for education and data analysis. Palaeontologia Electronica 4: 4-9:

Hansson BS, Stensmyr MC (2011) Evolution of Insect Olfaction. Neuron 72:698–711. doi: 10.1016/j.neuron.2011.11.003

Harrington LC, Scott TW, Lerdthusnee K, et al (2005) Dispersal of the dengue vector *Aedes aegypti* within and between rural communities. American Journal of Tropical Medicine and Hygiene 72: 209-220

Hassanali A, Herren H, Khan ZR, Pickett JA, Woodcock CM (2008) Integrated pest management: the push-pull approach for controlling insect pests and weeds of cereals, and its potential for other agricultural systems inlcuding animal husbandry. Philosophical Transaction of the Royal Society B: Biological Sciences 363: 611-621

Heard SB (1994) Imperfect oviposition decisions by the pitcher plant mosquito (*Wyeomyia smithii*). Evolutionary Ecology 8:493–502

Heisenberg M (1998) What do the mushroom bodies do for the insect brain? An introduction. Learning & Memory 5:1–10

Heisswolf A, Obermaier E, Poethke HJ (2005) Selection of large host plants for oviposition by a monophagous leaf beetle: nutritional quality or enemy-free space? Ecological Entomology 30:299–306. doi: 10.1111/j.0307-6946.2005.00706.

Hieu TT, Jung J, Kim S-I, et al (2014) Behavioural and electroantennogram responses of the stable fly (*Stomoxys calcitrans* L.) to plant essential oils and their mixtures with attractants: Effects of repellent oil with attractants on stable fly. Pest Management Science 70:163–172. doi: 10.1002/ps.3547

Higgins A (1986) The camel in health and disease. Baillière Tindall London

Himeidan YE, Temu EA, El Rayah EA, et al (2013) Chemical cues for malaria vectors oviposition site selection: Challenges and opportunities. Journal of Insects 2013:1–9. doi: 10.1155/2013/685182

Hoffmann AA, Ratna E, Sgrò CM, et al (2007) Antagonistic selection between adult thorax and wing size in field released *Drosophila melanogaster* independent of thermal conditions. Journal of Evolutionary Biology 20:2219–2227. doi: 10.1111/j.1420-9101.2007. 01422.x

Hogsette JA, Ruff J P (1986). Evaluation of flucythrinate- and fenvalerate-impregnated ear tags and permethrin ear tapes for fly (Diptera: Muscidae) control on beef and dairy cattle in Northwest Florida. Journal of Economic Entomology. 79: 152-157.

Hopkins AD (1916). Economic investigations of the scolytid bark and timber beetles of North America. U.S. Department of Agriculture Program of Work for 1917, p. 353

Hout MC, Papesh MH, Goldinger SD (2013) Multidimensional scaling. Wiley Interdisciplinary Reviews: Cognitive Science 4:93–103. doi: 10.1002/wcs.1203

Hu P, Li H, Zhang H, et al (2018) Experience-based mediation of feeding and oviposition behaviors in the cotton bollworm: *Helicoverpa armigera* (Lepidoptera: Noctuidae). PLOS ONE 13:e0190401. doi: 10.1371/journal.pone.0190401

Hugh-Jones M, Blackburn J (2009) The ecology of *Bacillus anthracis*. Molecular Aspects of Medicine 30: 356–367.

Hugh-Jones M, Blackburn J (2009) The ecology of *Bacillus anthracis*. Molecular Aspects of Medicine 30: 356–367.

Huotari M (2004) Odour sensing by insect olfactory receptor neurons: measurements of odours based on action potential analysis. University of Oulu

Ignell R, Dekker T, Ghaninia M, Hansson BS (2005) Neuronal architecture of the mosquito deutocerebrum. Journal of Comparative Neurology. 493, 207–240.

Iyengar A, Chakraborty TS, Goswami SP, et al (2010) Post-eclosion odor experience modifies olfactory receptor neuron coding in *Drosophila*. Proceedings of the National Academy of Sciences of the United States of America 107:9855–9860. doi: 10.1073/pnas.1003856107

Jaenike J (1978) On optimal oviposition behavior in phytophagous insects. Theoretical Population Biology 14:350–356. doi: 10.1016/0040-5809(78)90012-6

Jaenike J (1983) Induction of host preference in *Drosophila melanogaster*. Oecologia 58:320–325. doi: 10.1007/BF00385230

Jalil M, Rodriguez JG (1970) Studies of behaviour of *Macrocheles muscaedomesticae* (Acarina: Macrochelidae) with emphasis on its attraction to the house fly. Annals of the Entomological Society of America 63: 738–744.

Jantzen B, Eisner T (2008) Hindwings are unnecessary for flight but essential for execution of normal evasive flight in Lepidoptera. Proceedings of the National Academy of Sciences 105:16636–16640. doi: 10.1073/pnas.0807223105

Janz N, SöDerlind L, Nylin S (2009) No effect of larval experience on adult host preferences in *Polygonia c-album* (Lepidoptera: Nymphalidae): on the persistence of Hopkins′ host selection principle. Ecological Entomology 34:50–57. doi: 10.1111/j.1365-2311.2008.01041.

Jeanbourquin P (2005) The role of odour perception in the sensory ecology of the stable fly, *Stomoxys calcitrans* L. Université de Neuchâtel

Jeanbourquin P, Guerin PM (2007) Chemostimuli implicated in selection of oviposition substrates by the stable fly *Stomoxys calcitrans*. Medical and veterinary entomology 21:209–216

Jirakanjanakit N, Dujardin J-P (2005) Discrimination of *Aedes aegypti* (Diptera: Culicidae) laboratory lines based on wing geometry. The Southeast Asian Journal of Tropical Medicine public health 36:4

Johansson F, SöDerquist M, Bokma F (2009) Insect wing shape evolution: independent effects of migratory and mate guarding flight on dragonfly wings: Dragonfly wing shape evolution. Biological Journal of the Linnean Society 97:362–372. doi: 10.1111/j.1095-8312.2009. 01211.x

Johnson G, Panella N, Hale K, Komar N (2010) Detection of west nile virus in stable flies (Diptera: Muscidae) parasitizing juvenile American white pelicans. Journal of Medical Entomology 47: 1205–1211.

Jones CJ, Weinzierl RA (1997) Geographical and temporal variation in pteromalid (Hymenoptera: Pteromalidae) parasitism of stable fly and house fly (Diptera: Muscidae) pupae collected from Illinois cattle feedlots. Environmental Entomology 26: 421-432.

Jong Z-W, Kassim NFA, Naziri MA, Webb CE (2017) The effect of inbreeding and larval feeding regime on immature development of *Aedes albopictus*. Journal of Vector Ecology 42:105–112. doi: 10.1111/jvec.12244

Kacsoh BZ, Lynch ZR, Mortimer NT, Schlenke TA (2013) Fruit flies medicate offspring after seeing parasites. Science 339:947–950. doi: 10.1126/science.1229625

Kain P, Boyle SM, Tharadra SK, et al (2013) Odour receptors and neurons for DEET and new insect repellents. Nature 502:507–512. doi: 10.1038/nature12594

Kamala Jayanthi PD, Kempraj V, Aurade RM, et al (2014) Specific volatile compounds from mango elicit oviposition in gravid *Bactrocera dorsalis* females. Journal of Chemical Ecology 40:259–266. doi: 10.1007/s10886-014-0403-7

Kamaruzaman NAC, Mašán P, Velásquez Y, et al (2018) *Macrocheles* species (Acari: Macrochelidae) associated with human corpses in Europe. Experimental and Applied Acarology 76:453–471. doi: 10.1007/s10493-018-0321-4

Karageorgi M, Bräcker LB, Lebreton S, et al (2017) Evolution of multiple sensory systems drives novel egg-laying behavior in the fruit pest *Drosophila suzukii*. Current Biology 27:847–853. doi: 10.1016/j.cub.2017.01.055

Kassambara, A. (2017) Practical guide to principal component methods in R. http//www.sthda.com. 170p

Kay BH, Muir LE (1998) *Aedes aegypti* survival and dispersal estimated by mark-release-recapture in northern Australia. American Journal of Tropical Medicine and Hygiene 58:277–282. doi: 10.4269/ajtmh.1998.58.277

Keawrayup S, Duvallet G, Sukonthabhirom S, Chareonviriyaphap T (2012) Diversity of *Stomoxys* spp. (Diptera: Muscidae) and diurnal variations of activity of *Stomoxys indicus* and *S. Calcitrans* in a farm, in Wang Nam Khiao District, Nakhon ratchasima Province, Thailand. Parasite 19:259–265. doi: 10.1051/parasite/2012193259

Kerpel SM, Moreira GRP (2005) Absence of learning and local specialization on host plant selection by *Heliconius erato*. Journal Insect Behavior 18:433–452. doi: 10.1007/s10905-005-3701-7

Khaliq A, Ahmad MH, Ullah R, Anas M (2015) Behavioral ecology of oviposition in insects-A dumpy. Journal of Agricultural Research 4: 1-7.

Khan Z, Midega CAO, Hooper A, Pickett J (2016) Push-Pull: Chemical ecology-based integrated pest management technology. Journal of Chemical Ecology 42:689–697. doi: 10.1007/s10886-016-0730-y

Kiflawi M, Blaustein L, Mangel M (2003) Oviposition habitat selection by the mosquito *Culiseta longiareolata* in response to risk of predation and conspecific larval density. Ecological Entomology 28:168–173

Kitthawee S, Rungsri N (2011) Differentiation in wing shape in the *Bactrocera tau* (Walker) complex on a single fruit species of Thailand. ScienceAsia 37:308-313

Klingenberg CP (2011) MorphoJ: an integrated software package for geometric morphometrics: Computer program note. Molecular Ecology Resources 11:353–357. doi: 10.1111/j.1755-0998.2010. 02924.x

Klingenberg CP, Barluenga M, Meyer (2002) A shape analysis of symmetric structures: quantifying variation among individuals and asymmetry. Evolution 56: 1909–1920

Klinner CF, König C, Missbach C, et al (2016) Functional olfactory sensory neurons housed in olfactory sensilla on the ovipositor of the Hawkmoth *Manduca sexta*. Frontiers in Ecology and Evolution 4:. doi: 10.3389/fevo.2016.00130

Köhncke A (2013) When and where to lay your eggs? Humboldt-Universität zu Berlin, Mathematisch-Naturwissenschaftliche Fakultät I.

Kreher SA, Mathew D, Kim J, Carlson JR (2008) Translation of sensory input into behavioral output via and olfactory system. Neuron 59:110–124. doi: 10.1016/j.neuron.2008.06.010

Krinsky WL (1976) Animal disease agents transmitted by horse flies and deer flies (Diptera: Tabanidae). Journal of Medical Entomology 13: 225–275.

Ladner DT, Altizer S (2005a) Oviposition preference and larval performance of North American monarch butterflies on four *Asclepias* species. Entomologia experimentalis et applicata 116:9–20

Laurence BR, Pickett JA (1985) An oviposition attractant pheromone in *Culex quinquefasciatus* Say (Diptera: Culicidae). Bulletin of Entomological Research 75:283. doi: 10.1017/S0007485300014371

Leal WS (2004) Pheromone reception. In: Schulz S (ed) The chemistry of pheromones and other semiochemicals II. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 1–36

Leal WS (2013) Odorant reception in insects: roles of receptors, binding proteins, and degrading enzymes. Annual Review of Entomology. 58, 373–391. doi: 10.1146/annurev-ento-120811 -153635

Lecomte C, Thibout E (1993) Pre- and post-imaginal experience in a specialist parasitoid *Diadromus pulchellus* (Hym.: Ichneumonidae). Entomophaga 38:175–184. doi: 10.1007/BF02372551

Lee T, Lee A, Luo L (1999) Development of the *Drosophila* mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast. Development. 126: 4065–4076.

Legros M, Lloyd AL, Huang Y, Gould F (2009) Density-dependent intraspecific competition in the larval stage of *Aedes aegypti* (Diptera: Culicidae): Revisiting the current paradigm. Journal of Medical Entomology 46:409–419. doi: 10.1603/033.046.0301

Lehane MJ (2005) The biology of blood-sucking in insects. Cambridge University Press

Lenth R (2016) lsmeans: Least-squares means. R Package Version 2.20-23.

Li G, Ishikawa Y (2004) Oviposition deterrents in larval frass of four *Ostrinia* species fed on an artificial diet. Journal of Chemical Ecology 30:1445–1456. doi: 10.1023/B: JOEC.0000037750.64844.4b

Li S-Q, Zhang Z-N (2006) Influence of larval frass extracts on the oviposition behaviour of *Monochamus alternatus* (Col., Cerambycidae). Journal of Applied Entomology 130:177–182. doi: 10.1111/j.1439-0418.2006. 01039.

Liaw A, Wiener M (2002) Classification and regression by randomForest. R news 2:18–22

Lin T, Li C, Liu J, Smith BH, Lei H, Zeng X (2018) Glomerular organization in the antennal lobe of the oriental fruit fly *Bactrocera dorsalis*. Frontiers in Neuroanatomy. 12: 71. doi: 10.3389/fnana.2018.00071

Lin X, Xu Y, Jiang J, et al (2018) Host quality induces phenotypic plasticity in a wing polyphenic insect. Proceedings of the National Academy of Sciences 115:7563–7568. doi: 10.1073/pnas.1721473115

Lindh JM, Nnaste AK, Knols BGJ, Faye I (2008) Oviposition responses of *Anopheles gambiae* s.s. (Diptera: Culicidae) and identification of volatiles from Bacteria-containing solutions. Journal of Medical Entomology 45:11

Lindh JM, Okal MN, Herrera-Varela M, et al (2015) Discovery of an oviposition attractant for gravid malaria vectors of the *Anopheles gambiae* species complex. Malaria Journal 14:. doi: 10.1186/s12936-015-0636-0

Linz J, Baschwitz A, Strutz A, et al (2013) Host plant-driven sensory specialization in *Drosophila erecta*. Proceedings of the Royal Society B: Biological Sciences 280:20130626. doi: 10.1098/rspb.2013.0626

Liu S-S, Liu T-X (2006) Preimaginal conditioning does not affect oviposition preference in the diamondback moth. Ecological Entomology 31:307–315. doi: 10.1111/j.1365-2311.2006.00777.x

Logan JG, Birkett MA (2007) Semiochemicals for biting fly control: their identification and exploitation. Pest Management Science 63:647–657. doi: 10.1002/ps.1408

Lorenz C, Almeida F, Almeida-Lopes F, et al (2017) Geometric morphometrics in mosquitoes: what has been measured? Infection, Genetics and Evolution 54:205–215. doi: 10.1016/j.meegid.2017.06.029

Lu T, Qiu, YT, Wang G, Kwon, JY, Rützler, M, Kwon, H, Pitts, RJ, van Loon, JJA., Takken W, Carlson JR, Zwiebel LJ (2007). Odor coding in the maxillary palp of the malaria vector mosquito *Anopheles gambiae*. Current Biology 17: 1533–1544.

Luong LT, Penoni LR, Horn CJ, Polak M (2015) Physical and physiological costs of ectoparasitic mites on host flight endurance. Ecological Entomology 40:518–524. doi: 10.1111/een.12218

Lyu F, Hai X, Wang Z, Bi Y (2018) Influence of visual cues on oviposition site searching and learning behavior in the parasitic beetle *Dastarcus helophoroides* (Fairmaire) (Coleoptera: Bothrideridae). Scientific Reports 8:. doi: 10.1038/s41598-018-35580-4

Machtinger ET, Geden CJ, Hogsette JA, Leppla NC (2014) Development and oviposition preference of house flies and stable flies (Diptera: Muscidae) in six substrates from Florida Equine Facilities. Journal of Medical Entomology 51:1144–1150. doi: 10.1603/ME14040

Mansourian S, Corcoran J, Enjin A, et al (2016) Fecal-derived phenol induces egg-laying aversion in *Drosophila*. Current Biology 26:2762–2769. doi: 10.1016/j.cub.2016.07.065

Marayati BF, Schal C, Ponnusamy L, et al (2015) Attraction and oviposition preferences of *Phlebotomus papatasi* (Diptera: Psychodidae), vector of Old-World cutaneous leishmaniasis, to larval rearing media. Parasites & Vectors 8:. doi: 10.1186/s13071-015-1261-z

Margulies C, Tully T, Dubnau J (2005) Deconstructing memory in *Drosophila*. Current Biology 15:R700–R713. doi: 10.1016/j.cub.2005.08.024

Marneweck C, Jürgens A, Shrader AM (2017a) Dung odours signal sex, age, territorial and oestrous state in white rhinos. Proceedings of the Royal Society B: Biological Sciences 284:20162376. doi: 10.1098/rspb.2016.2376

Marneweck C, Jürgens A, Shrader AM (2017b) Temporal Variation of White Rhino Dung Odours. Journal of Chemical Ecology 43:955–965. doi: 10.1007/s10886-017-0890-4

Masmeatathip R, Gilles J, Ketavan C, Duvallet G (2006a) First survey of seasonal abundance and daily activity of *Stomoxys* spp. (Diptera: Muscidae) in Kamphaengsaen Campus, Nakornpathom province, Thailand. Parasite 13:245–250. doi: 10.1051/parasite/2006133245

Masmeatathip R, Ketavan C, Duvallet G (2006c) Morphological studies of *Stomoxys* spp. (Diptera: Muscidae) in central Thailand. Kasetsart J(Nat Sci) 40:872–881

Mansourian S, Corcoran J, Enjin A, et al (2016) Fecal-derived phenol induces egg-laying aversion in *Drosophila*. Current Biology 26:2762–2769. doi: 10.1016/j.cub.2016.07.065

Matthiessen MK, Larney FJ, Brent Selinger L, Olson AF (2005) Influence of loss-on-ignition temperature and heating time on ash content of compost and manure. communications in soil science and plant analysis 36:2561–2573. doi: 10.1080/00103620500257242

Matthew K, Lau. DTK (2013) Dunnett-Tukey-Kramer pairwise multiple comparison test adjusted for unequal variances and unequal sample sizes. R package version 3.5. https://CRAN.R-project.org/package=DTK.

Mavoungou JF, Mintsa Nguema R, Lydie Acapovi G, et al (2017) Breeding sites of *Stomoxys* spp (Diptera: Muscidae), a preliminary study in the Makokou region (North-East-Gabon). Vector Biology Journal 02: doi: 10.4172/2473-4810.1000115

McCall PJ, Eaton G (2001) Olfactory memory in the mosquito *Culex quinquefasciatus*. Medical and Veterinary Entomology 15:197–203. doi: 10.1046/j.0269-283x.2001.00304.

McDonald JH (2009) Handbook of biological statistics. Sparky House Publishing Baltimore, MD

McCormick AC, Reinecke A, Gershenzon J, Unsicker SB (2016) Feeding experience affects the behavioral response of polyphagous gypsy moth caterpillars to herbivore-induced poplar volatiles. Journal of Chemical Ecology 42:382–393. doi: 10.1007/s10886-016-0698-7

McIver SB (1982) Sensilla of mosquitoes (Diptera: Culicidae). Journal of Medical. Entomology. 19, 489–535.

Menda G, Uhr JH, Wyttenbach RA, et al (2013) Associative learning in the dengue vector mosquito, *Aedes aegypti*: avoidance of a previously attractive odor or surface color that is paired with an aversive stimulus. The Journal of Experimental Biology 216:218–223. doi: 10.1242/jeb.074898

Menger DJ, Otieno B, De Rijk M, Mukabana RW, Van Loon JJ, Takken W (2014) Apush-pull system to reduce house entry of malaria mosquitoes. Malaria Journal 13: 119

Michaud JP, Jyoti JL (2007) Repellency of conspecific and heterospecific larval residues to *Hippodamia convergens* (Coleoptera: Coccinellidae) ovipositing on sorghum plants. European Journal of Entomology 104:399–405. doi: 10.14411/eje.2007.059

Mihok S, Kang'ethe EK, Kamau GK (1995) Trials of traps and attractants for *Stomoxys* spp. (Diptera: Muscidae). Journal of Medical Entomology 32:283–289. doi: 10.1093/jmedent/32.3.283

Mihok S, Munyoki E, Saleh K (1996) Phenology of Stomoxyinae in a kenyan forest. Medical and Veterinary Entomology 10:305–316. doi: 10.1111/j.1365-2915. 1996.tb00750.

Meyer A, Galizia CG, Nawrot MP (2013) Local interneurons and projection neurons in the antennal lobe from a spiking point of view. Journal of Neurophysiology 110: 2465–2474.

Molnár BP, Tóth Z, Kárpáti Z (2017) Synthetic blend of larval frass volatiles repel oviposition in the invasive box tree moth, *Cydalima perspectalis*. Journal of Pest Science 90:873–885. doi: 10.1007/s10340-017-0837-0

Moraes APR, Angelo I da C, Fernandes ÉKK, et al (2008) Virulence of *Metarhizium anisopliae* to eggs and immature stages of *Stomoxys calcitrans*. Annals of the New York Academy of Sciences 1149:384–387. doi: 10.1196/annals.1428.008

Motoki MT, Suesdek L, Bergo ES, Sallum MAM (2012) Wing geometry of *Anopheles darlingi* Root (Diptera: Culicidae) in five major Brazilian ecoregions. Infection, Genetics and Evolution 12:1246–1252. doi: 10.1016/j.meegid.2012.04.002

Mount GA, Lofgren CS, Gahan JB (1966) Malathion, naled, Fenthion, and Bayer 39007 thermal fogs for control of the stable fly (dog fly), *Stomoxys calcitrans* (Diptera: Muscidae). Florida Entomologist 49: 169-173

Mousseau T (1998) The adaptive significance of maternal effects. Trends in Ecology & Evolution 13:403–407. doi: 10.1016/S0169-5347(98)01472-4

Nagai T (1983) On the relationship between the electroantennogram and simultaneously recorded single sensillum response of the european corn borer, *Ostrinia nubilalis*. Archives of Insect Biochemistry and Physiology 1:85–91. doi: 10.1002/arch.940010109

Nakagawa T, Sakurai T, Nishioka T, and Touhara K (2005) Insect sex pheromone signals mediated by specific combinations of olfactory receptors. Science 307: 1638–1642.

Neuser K, Husse J, Stock P, Gerber B (2005) Appetitive olfactory learning in *Drosophila* larvae: effects of repetition, reward strength, age, gender, assay type and memory span. Animal Behaviour 69:891–898. doi: 10.1016/j.anbehav.2004.06.013

Ning SY, Yang HY, Fan DS, Feng JN (2018) Influence of larval experience on preference of a subterranean insect *Delia antiqua* on *Allium* hosts. Journal of Applied Entomology 142:263–271. doi: 10.1111/jen.12464

Njiru ZK, Ole-Mapeny JO, Ouma JM, Ndung'u W, Olaho Mukani IM (2001). Prevalence of trypanosomosis in camel calves: a pilot study in Laikipia District of Kenya. Revue d'élevage et de médecine vétérinaire des pays tropicaux 34:183186.

Norin T (2007) Semiochemicals for insect pest management. Pure and Applied Chemistry 79: doi: 10.1351/pac200779122129

Notter-Hausmann C, Dorn S (2010) Relationship between behavior and physiology in an invasive pest species: Oviposition site selection and temperature-dependent development of the oriental fruit moth (Lepidoptera: Tortricidae). Environmental entomology 39:561–569. doi: 10.1603/EN09231

Obermayr U, Ruther J, Bernier UR, Rose A, Geier (2015) Evaluation of push-oull system approach for *Aedes aegypti* (L.) using a novel dispensing system for spatial repellents in the laboratory and in a semi-field environnement. PloS ONE 10: 1-18

Oguz G, Kasap OE, Alten B (2017) Wing morphology variations in a natural population of *Phlebotomus tobbi* Adler and Theodor 1930. Journal of Vector Ecology 42:223–232. doi: 10.1111/jvec.12262

Oliveira MT, Barau JG, Junqueira ACM, et al (2008) Structure and evolution of the mitochondrial genomes of *Haematobia irritans* and *Stomoxys calcitrans*: The Muscidae (Diptera: Calyptratae) perspective. Molecular Phylogenetics and Evolution 48:850–857. doi: 10.1016/j.ympev.2008.05.022

Olsson P-OC, Anderbrant O, Löfstedt C (2006) Experience influences oviposition behaviour in two pyralid moths, *Ephestia cautella* and *Plodia interpunctella*. Animal Behaviour 72:545–551. doi: 10.1016/j.anbehav.2005.10.023

Olsson SB, Hansson BS (2013) Electroantennogram and single sensillum recording in Insect antennae. In: Touhara K (ed) Pheromone signaling. Humana Press, Totowa, NJ, pp 157–177

Onagbola EO, Fadamiro HY (2008) Scanning electron microscopy studies of antennal sensilla of *Pteromalus cerealellae* (Hymenoptera: Pteromalidae). Micron 39:526–535. doi: 10.1016/j.micron.2007.08.001

Özbek HH, Bal DA, Doğan S (2015) The genus *Macrocheles* Latreille (Acari: Mesostigmata: Macrochelidae) from Kelkit Valley (Turkey), with three newly recorded mite species. Turkish Journal of Zoology 39:768–780. doi: 10.3906/zoo-1409-14

Pagadala Damodaram KJ, Kempraj V, Aurade RM, et al (2014) Oviposition site-selection by *Bactrocera dorsalis* is mediated through an innate recognition template tuned to γ-Octalactone. PLoS ONE 9: e85764. doi: 10.1371/journal.pone.0085764

Pagadala Damodaram KJ, Kempraj V, Aurade RM, et al (2014) Oviposition site-selection by *Bactrocera dorsalis* is mediated through an innate recognition template tuned to γ-Octalactone. PLoS ONE 9: e85764. doi: 10.1371/journal.pone.0085764

Panigrahi SK, Barik TK, Mohanty S, Tripathy NK (2014) Laboratory evaluation of oviposition behavior of field collected *Aedes* mosquitoes. Journal of Insects 2014:1–8. doi: 10.1155/2014/207489

Pellegrino M (2011) Structure-function analysis of insect olfactory receptors. Student Theses and Dissertations. Paper 151

Pelosi P, Zhou J-J, Ban LP, Calvello M (2006) Soluble proteins in insect chemical communication. Cellular and Molecular Life Sciences 63:1658–1676. doi: 10.1007/s00018-005-5607-0

Perkins MC, Woods HA, Harrison JF, Elser JJ (2004) Dietary phosphorus affects the growth of larval *Manduca sexta*. Archives of Insect Biochemistry and Physiology 55:153–168. doi: 10.1002/arch.10133

Pers JNCV der, Minks AK (1993) Pheromone monitoring in the field using single sensillum recording. Entomologia Experimentalis et Applicata 68:237–245. doi: 10.1111/j.1570-7458. 1993.tb01709

Perry JN., Wall C, Greenway AR (1980) Latin square designs in field experiments involving insect sex attractants. Ecological Entomology 5 385-396.

Pieterse W, Benítez HA, Addison P. (2017) The use of geometric morphometric analysis to illustrate the shape change induced by different fruit hosts on the wing shape of *Bactrocera dorsalis* and Ceratitis capitata (Diptera: Tephritidae). Zoologischer Anzeiger 269:110–6.

Pitzer JB (2010) The ecology of stable flies (Diptera: Muscidae) associated with equine facilities in Florida. University of Florida

Pitzer JB, Kaufman PE, Tenbroeck SH (2010) Assessing permethrin resistance in the stable fly (Diptera: Muscidae) in florida by using laboratory selections and field evaluations. Journal of Economic Entomology 103:2258–2263. doi: 10.1603/EC10166

Pitzer JB, Kaufman PE, Tenbroeck SH, Maruniak JE (2011) Host blood meal identification by multiplex polymerase chain reaction for dispersal evidence of stable flies (Diptera: Muscidae) between livestock facilities. Journal of Medical Entomology 48:53–60. doi: 10.1603/ME10123

Polak M (1996) Ectoparasitic effects on host survival and reproduction: The Drosophila--Macrocheles association. Ecology 77:1379–1389. doi: 10.2307/2265535

Polak M, Markow TA (1995) Effect of ectoparasitic mites on sexual selection in a Sonoran Desert fruit fly. Evolution 49:660–669. doi: 10.1111/j.1558-5646. 1995.tb02302.

Prokopy RJ, Roitberg BD (2001) Joining and avoidance behaviour in social insects. Annual Review of Entomology 46:631–665. doi: 10.1146/annurev.ento.46.1.631

Proffit M, Khallaf MA, Carrasco D, et al (2015) 'Do you remember the first time?' Host plant preference in a moth is modulated by experiences during larval feeding and adult mating. Ecology Letters 18:365–374. doi: 10.1111/ele.12419

Prudic KL, Oliver JC, Bowers MD (2005) Soil nutrient effects on oviposition preference, larval performance, and chemical defense of a specialist insect herbivore. Oecologia 143:578–587. doi: 10.1007/s00442-005-0008-5

R Core Team. R: (2018) A language and environment for statistical computing R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Raitanen J, Forsman JT, Kivelä SM, et al (2014) Attraction to conspecific eggs may guide oviposition site selection in a solitary insect. Behavioral Ecology 25:110–116. doi: 10.1093/beheco/art092

Ranganathan Y, Borges RM (2010) Reducing the babel in plant volatile communication: using the forest to see the trees: Random Forest-based volatile selection. Plant Biology 12:735–742. doi: 10.1111/j.1438-8677.2009.00278.

Ray S (1999) Survival of olfactory memory through metamorphosis in the fly *Musca domestica*. Neuroscience Letters 259:37–40. doi: 10.1016/S0304-3940(98)00892-1

Reh-Hamburg (1924) Hopkins host-selection principle as related to certain Cerambycid beetles. Zeitschrift für Induktive Abstammungs- und Vererbungslehre 32:89–91. doi: 10.1007/BF01816748

Reisenman CE, Lei H, Guerenstein PG (2016) Neuroethology of olfactory-guided behavior and its potential application in the control of harmful insects. Frontiers in Physiology 7: doi: 10.3389/fphys.2016.00271

Reiskind MH, Greene KL, Lounibos LP (2009) Leaf species identity and combination affect performance and oviposition choice of two container mosquito species. Ecological Entomology 34:447–456. doi: 10.1111/j.1365-2311.2008.01067.

Reiskind MH, Lounibos LP (2009) Effects of intraspecific larval competition on adult longevity in the mosquitoes *Aedes aegypti* and *Aedes albopictus*. Medical and Veterinary Entomology 23:62–68. doi: 10.1111/j.1365-2915.2008.00782.

Reiskind MH, Wilson ML (2004) *Culex restuans* (Diptera: Culicidae) oviposition behavior determined by larval habitat quality and quantity in south-eastern Michigan. Journal of Medical Entomology 41:179–186. doi: 10.1603/0022-2585-41.2.179

Resetarits Jr WJ (1996) Oviposition site choice and life history evolution. American Zoologist 36:205–215

Ribak G, Barkan S, Soroker V (2017) The aerodynamics of flight in an insect flight-mill. PLOS ONE 12: e0186441. doi: 10.1371/journal.pone.0186441

Rietdorf K, Steidle JLM (2002) Was hopkins right? influence of larval and early adult experience on the olfactory response in the granary weevil *Sitophilus granarius* (Coleoptera, Curculionidae). Physiological Entomology 27:223–227. doi: 10.1046/j.1365-3032.2002.00289.x

Riffell JA, Shlizerman E, Sanders E, et al (2014) Flower discrimination by pollinators in a dynamic chemical environment. Science 344:1515–1518. doi: 10.1126/science.1251041

Rizvi SZM, Raman A (2017) Effect of leaf chemistry of *Vitis vinifera* L. on the performance and development of *Epiphyas postvittana* (Lepidoptera: Tortricidae): Effect of grape cultivar on *Epiphyas postvittana* performance. Australian Journal of Grape and Wine Research 23:95–102. doi: 10.1111/ajgw.12244

Roberts D (2014) Mosquito larvae change their feeding behavior in response to kairomones from some predators. Journal of Medical Entomology 51:368–374. doi: 10.1603/ME13129

Rodriguez JG, Singh P, Taylor B (1970) Manure mites and their role in fly control. Journal of Medical Entomology **7**: 335–41.

Roelofs WL (1984) Electroantennogram assays: Rapid and convenient screening procedures for pheromones. In: Hummel HE, Miller TA (eds) Techniques in pheromone research. Springer New York, New York, NY, pp 131–159

Rojas JC, Wyatt TD (1999) The role of pre- and post-imaginal experience in the host-finding and oviposition behaviour of the cabbage moth. Physiological Entomology 24:83–89. doi: 10.1046/j.1365-3032.1999.00117.

Romero A, Broce A, Zurek L (2006) Role of bacteria in the oviposition behaviour and larval development of stable flies. Medical and veterinary entomology 20:115–121

Sadek MM, Hansson BS, Anderson P (2010) Does risk of egg parasitism affect choice of oviposition sites by a moth? A field and laboratory study. Basic and Applied Ecology 11:135–143. doi: 10.1016/j.baae.2009.09.003

Sadtler PT, Quick KM, Golub MD, et al (2014) Neural constraints on learning. Nature 512:423–426. doi: 10.1038/nature13665

Salem A (2012) *Stomoxys calcitrans* (L. 1758): morphologie, biologie, rôle vecteur et moyens de lutte

Salem A, Franc M, Jacquiet P, et al (2012) Feeding and breeding aspects of *Stomoxys calcitrans* (Diptera: Muscidae) under laboratory conditions. Parasite 19:309–317

Sanes, JR, Hildebrand JG (1976) Structure and development of antennae in a moth, *Manduca sexta*. Developmental Biology. 51: 280–299.

Sandoval Ramirez CM, Nieves Blanco EE, Gutiérrez Marin R, et al (2015) Morphometric analysis of the host effect on phenotypical variation of *Belminus ferroae* (Hemiptera: Triatominae). Psyche: A Journal of Entomology 2015:1–12. doi: 10.1155/2015/613614

Santana AK, Zucoloto F (2011) Influence of previous experience on the preference, food utilization and performance of *Ascia monuste orseis* wild larvae (Godart) (Lepidoptera: Pieridae) for three different hosts. Neotropical Entomology 40:631-638.

Sato K, Pellegrino M, Nakagawa T, Nakagawa T, Vosshall LB, and Touhara K (2008). Insect olfactory receptors are heteromeric ligand gated ion channels. Nature 452: 1002–1006.

Sendaydiego JP, Demayo CG (2015) Describing variations in wing shapes of *Anopheles flavirostris* detected positive and negative of filaria using relative warp and Euclidean distance matrix analysis. International Journal of Mosquito Research 2: 09-13

Scheidler NH, Liu C, Hamby KA, et al (2015) Volatile codes: correlation of olfactory signals and reception in *Drosophila*-yeast chemical communication. Scientific Reports 5: doi: 10.1038/srep14059

Schneider D, Boeckh J (1962) Rezeptorpotential und nervenimpulse einzelner olfaktorischer sensillen der insektenantenne. Zeitschrift für vergleichende Physiologie 45:405–412

Schofield S, Cork A, Brady J (1995) Electroantennogram responses of the stable fly, *Stomoxys calcitrans*, to components of host odour. Physiological Entomology. 20: 273-280.

Schofield RMS, Nesson MH, Richardson KA (2002) Tooth hardness increases with zinc-content in mandibles of young adult leaf-cutter ants. Naturwissenschaften 89: 579-83

Schowalter TD, Klowden MJ (1979) Blood meal size of the stable fly, *Stomoxys calcitrans* measured by the HiCN method. Mosquitoe News 9:110-112.

Schwartz NU, Zhong L, Bellemer A, Tracey WD (2012) Egg laying decisions in *Drosophila* are consistent with foraging costs of larval progeny. PLoS ONE 7:e37910. doi: 10.1371/journal.pone.0037910

Seada MA (2015) Antennal morphology and sensillum distribution of female cotton leaf worm *Spodoptera littoralis* (Lepidoptera: Noctuidae). The Journal of Basic & Applied Zoology 68:10–18. doi: 10.1016/j.jobaz.2015.01.005

Seenivasagan T, Sharma KR, Sekhar K, et al (2009) Electroantennogram, flight orientation, and oviposition responses of *Aedes aegypti* to the oviposition pheromone n-heneicosane. Parasitology Research 104:827–833. doi: 10.1007/s00436-008-1263-2

Shanbhag SR, Müller, B, Steinbrecht, RA (1999) Atlas of olfactory organs of *Drosophila melanogaster*-1. Types, external organization, innervation and distribution of olfactory sensilla. International Journal Insect Morphology Embryology. 28, 377–397.

Shapiro LLM, Murdock CC, Jacobs GR, et al (2016) Larval food quantity affects the capacity of adult mosquitoes to transmit human malaria. Proceedings of the Royal Society B: Biological Sciences 283:20160298. doi: 10.1098/rspb.2016.0298

Sharif S, Jacquiet P, Prevot F, et al (2019) *Stomoxys calcitrans*, mechanical vector of virulent *Besnoitia besnoiti* from chronically infected cattle to susceptible rabbit. Medical and Veterinary Entomology 0: doi: 10.1111/mve.12356

Shields VD, Heinbockel T (2012) Neurophysiological recording techniques applied to insect chemosensory systems. INTECH Open Access Publisher

Shimmi O, Matsuda S, Hatakeyama M (2014) Insights into the molecular mechanisms underlying diversified wing venation among insects. Proceedings of the Royal Society B: Biological Sciences 281:20140264–20140264. doi: 10.1098/rspb.2014.0264

Silberbush A, Markman S, Lewinsohn E, et al (2010) Predator-released hydrocarbons repel oviposition by a mosquito. Ecology Letters 13:1129–1138. doi: 10.1111/j.1461-0248.2010.01501.

Silbering AF, Benton R (2010) Ionotropic and metabotropic mechanisms in chemoreception: 'chance or design'? EMBO reports 11:173–179. doi: 10.1038/embor.2010.8

Sivakumar R, Jebanesan A, Govindarajan M, Rajasekar P (2011). Oviposition attractancy of dodecanoic, hexadecanoic and tetradecanoic acids against *Aedes aegypti* and *Culex quinquefasciatus* (Diptera: Culicidae). European Review for Medical and Pharmacological Sciences 15: 1172-1175.

Skovgård H, Jespersen JB (1999) Activity and relative abundance of hymenopterous parasitoids that attack puparia of *Musca domestica* and *Stomoxys calcitrans* (Diptera: Muscidae) on confined pig and cattle farms in Denmark. Bulletin of Entomological Research 89: 263-26.

Sladecek FXJ, Segar ST, Lee C, et al (2017) Temporal segregation between dung-inhabiting beetle and fly species. PLOS ONE 12: e0170426. doi: 10.1371/journal.pone.0170426

Solari P, Corda V, Sollai G, Kreissl S, Galizia CG, Crnjar R (2016). Morphological characterization of the antennal lobes in the Mediterranean fruit fly *Ceratitis capitata*. J. Comp. Physiol. A. 202, 131–146.

Solarz SL, Newman RM (2001) Variation in hostplant preference and performance by the milfoil weevil, *Euhrychiopsis lecontei* Dietz, exposed to native and exotic watermilfoils. Oecologia 126:66–75. doi: 10.1007/s004420000484

Solórzano J-A, Gilles J, Bravo O, et al (2015) Biology and trapping of stable Flies (Diptera: Muscidae) developing in pineapple residues (*Ananas comosus)* in Costa Rica. Journal of Insect Science 15:145. doi: 10.1093/jisesa/iev127

Somme L (1985) The number of stable flies in Norwegian barns and their resistance to DDT. Journal of Economic Entomology 51: 599–601

Sommer C, Steffansen B, Nielsen BO, Grønvold J, Vagn Jensen K.-M, Brochner Jespersen, J, Springborg J, Nansen P (1992) Ivermectin excreted in cattle dung after subcutaneous injection or pour-on treatment: concentrations and impact on dung fauna Bulletin of Entomological Research, 82: 257-264.

Soto IM, Carreira VP, Soto EM, Hasson E (2008) Wing morphology and fluctuating asymmetry depend on the host plant in cactophilic *Drosophila*. Journal of Evolutionary Biology 21:598–609. doi: 10.1111/j.1420-9101.2007.01474.

Späthe A, Reinecke A, Olsson SB, et al (2013) Plant species- and status-specific odorant blends guide oviposition choice in the moth *Manduca sexta*. Chemical Senses 38:147–159. doi: 10.1093/chemse/bjs089

Städler E (1994) Oviposition behavior of insects influenced by chemoreceptors. In: Kurihara K, Suzuki N, Ogawa H (eds) Olfaction and taste XI. Springer Japan, Tokyo, pp 821–826

Staley JT, Stewart-Jones A, Poppy GM, et al (2009) Fertilizer affects the behaviour and performance of *Plutella xylostella* on brassicas. Agricultural and Forest Entomology 11:275–282. doi: 10.1111/j.1461-9563.2009.00432.

Stengl M, Funk NW (2013) The role of the coreceptor Orco in insect olfactory transduction. Journal of Comparative Physiology 199: 897–909.

Stephens CR, Juliano SA (2012) Wing shape as an indicator of larval rearing conditions for *Aedes albopictus* and *Aedes aegypti* (Diptera: Culicidae). Journal of Medical Entomology 49:927–938. doi: 10.1603/ME12012

Stoffolano JG, Yin LRS (1987) Structure and function of the ovipositor and associated sensilla of the apple maggot, *Rhagoletis pomonella* (Walsh) (Diptera: Tephritidae). International Journal of Insect Morphology and Embryology 16:41–69. doi: 10.1016/0020-7322(87)90055-9

Suesdek L, Lorenz C (2013) Evaluation of chemical preparation on insect wing shape for geometric morphometrics. The American Journal of Tropical Medicine and Hygiene 89:928–931. doi: 10.4269/ajtmh.13-0359

Sugiura S, Yamazaki K, Yamaura Y (2007) Intraspecific competition as a selective pressure on the choice of oviposition site in a phytophagous insect: Intraspecific competition of a leaf miner. Biological Journal of the Linnean Society 92:641–650. doi: 10.1111/j.1095-8312.2007.00857.

Sumba AL, Mihok S, Oyieke, FA (1998). Mechanical transmission of *Trypanosoma evansi* and *T. congolense* by *Stomoxys niger* and *S. taeniatus* in a laboratory mouse model. Medical and Veterinary Entomology 12: 417–422

Tangtrakulwanich K, Albuquerque TA, Brewer GJ, et al (2015) Behavioural responses of stable flies to cattle manure slurry associated odourants: Stable fly attracted to manure slurry volatiles. Medical and Veterinary Entomology 29:82–87. doi: 10.1111/mve.12103

Tangtrakulwanich K, Chen H, Baxendale F, et al (2011) Characterization of olfactory sensilla of S*tomoxys calcitrans* and electrophysiological responses to odorant compounds associated with hosts and oviposition media. Medical and Veterinary Entomology 25:327–336. doi: 10.1111/j.1365-2915.2011.00946.

Takken W, Klowden MJ, Chambers GM (1998) Articles: effect of body size on host seeking and blood meal utilization in *Anopheles gambiae* sensu stricto (Diptera: Culicidae): the disadvantage of being small. Journal of Medical Entomology 35:639–645. doi: 10.1093/jmedent/35.5.639

Tauxe GM, MacWilliam D, Boyle SM, et al (2013) Targeting a dual detector of skin and CO2 to modify mosquito host seeking. Cell 155:1365–1379. doi: 10.1016/j.cell.2013.11.013

Taylor DB, Moon RD, Campbell JB, et al (2010) Dispersal of stable flies (Diptera: Muscidae) from larval development sites in a Nebraska Landscape. Environmental Entomology 39:1101–1110. doi: 10.1603/EN10057

Taylor DB, Moon RD, Mark DR (2012) Economic impact of stable flies (Diptera: Muscidae) on dairy and beef cattle production. Journal of Medical Entomology 49:198–209. doi: 10.1603/ME10050

Tekle T, Abebe G (2001) Trypanosomosis and helminthoses: Major health problems of camels (*Camelus dromedaries*) in the Southern Rangelands of Borena, Ethiopians Journal of Camel Practical Research 8: 39-42.

Therneau T (2015) A package for survival analysis in S_. version 2.38, <URL: https://CR AN. R-project.org/package=survival.

Tholl D, Boland W, Hansel A, et al (2006) Practical approaches to plant volatile analysis. The Plant Journal 45:540–560. doi: 10.1111/j.1365-313X.2005.02612.

Traversa D, Otranto D, Iorio R, Carluccio A, Contri A, Paoletti B, Bartolini R, Giangaspero A (2008) Identification of the intermediate hosts of *Habronema microstoma* and *Habronema muscae* under field conditions. Medical and Veterinary Entomology 22: 283–287.

Trotta V, Duran Prieto J, Battaglia D, Fanti P (2014) Plastic responses of some life history traits and cellular components of body size in *Aphidius ervi* as related to the age of its host *Acyrthosiphon pisum*: Wing Shape Variation in *A. ervi*. Biological Journal of the Linnean Society 113:439–454. doi: 10.1111/bij.12354

Tsuchihara K, Fujikawa K, Ishiguro M, Yamada T, Tada C, Ozaki K, Ozaki M (2005) An odorant-binding protein facilitates odorant transfer from air to hydrophilic surroundings in the blowfly. Chemical Senses 30: 559–564.

Tully T, Cambiazo V, Kruse L (1994) Memory through metamorphosis in normal and mutant *Drosophila*. The Journal of Neuroscience 7

Tunnakundacha S, Desquesnes M, Masmeatathip R (2017) Comparison of vavoua, Malaise and Nzi traps with and without attractants for trapping of *Stomoxys* spp. (Diptera: Muscidae) and tabanids (Diptera: Tabanidae) on cattle farms. Agriculture and Natural Resources. doi: 10.1016/j.anres.2017.07.002

Valladares G, Lawton JH (1991) Host-Plant Selection in the Holly Leaf-Miner: Does Mother Know Best? The Journal of Animal Ecology 60:227. doi: 10.2307/5456

Venables WN, Ripley BD (2002) Modern applied statistics with S (4th ed). New York: Springer.

Venthur H, Zhou J-J (2018) Odorant receptors and odorant-binding proteins as insect pest control Targets: A comparative analysis. Frontiers in Physiology 9:. doi: 10.3389/fphys.2018.01163

Vidal PO, Suesdek L (2012) Comparison of wing geometry data and genetic data for assessing the population structure of *Aedes aegypti*. Infection, Genetics and Evolution 12:591–596. doi: 10.1016/j.meegid.2011.11.013

Vijendravarma RK, Narasimha S, Kawecki TJ (2013) Predatory cannibalism in *Drosophila melanogaster* larvae. Nature Communications 4: doi: 10.1038/ncomms2744

Virginio F, Oliveira Vidal P, Suesdek L (2015) Wing sexual dimorphism of pathogen-vector culicids. Parasites & Vectors 8: doi: 10.1186/s13071-015-0769-6

Viscosi V, Cardini A (2011) Leaf morphology, taxonomy and geometric morphometrics: A Simplified Protocol for Beginners. PLoS ONE 6: e25630. doi: 10.1371/journal.pone.0025630

Vosshall LB, Stocker RF (2007). Molecular architecture of smell and taste in *Drosophila*. Annual Review of Neuroscience. 30: 505–533.

Vosshall LB, Wong AM, Axel R (2000) An olfactory sensory map in the fly brain. Cell 102: 147–159.

Wachira SW, Ndung'u M, Njagi PGN, Hassanali A (2010) Comparative responses of ovipositing *Anopheles gambiae* and *Culex quinquefasciatus* females to the presence of *Culex* egg rafts and larvae. Medical and Veterinary Entomology 24:369–374. doi: 10.1111/j.1365-2915.2010.00913.

Wagman MJ, Grieco PJ, Bautista K, Polanco J, Briceno I, King R, Achee LN (2015) The evaluation of a push-pull system to control malaria vectors in northern Belize, Central America.Malaria Journal. 14: 1-11.

Walker AR (1990). Disease caused by arthropods. In Sewell MMH, Brocklesby DW (Eds.), Handbook on Animal Diseasesin the Tropics (4th edition), Bailliere Tindall: London.

Wang Y (2017) Higher plasticity in feeding preference of a generalist than a specialist: experiments with two closely related *Helicoverpa* species. Scientific Reports 12

Warton DI, Hui FK (2011) The arcsine is asinine: the analysis of proportions in ecology. Ecology 92:3–10

Watson DW, Geden CJ, Long SJ, Rutz DA (1995) Efficacy of *Beauveria bassiana* for controlling the house fly and stable fly (Diptera: Muscidae). Biological Control 5: 405-411.

Weeks ENI, Machtinger ET, Gezan SA, et al (2016) Effects of four commercial fungal formulations on mortality and sporulation in house flies (*Musca domestica*) and stable flies (*Stomoxys calcitrans*): Effects of fungal formulations in filth flies. Medical and Veterinary Entomology. doi: 10.1111/mve.12201

West-Eberhard MJ (2005) Developmental plasticity and the origin of species differences. Proceedings of the National Academy of Sciences 102:6543–6549. doi: 10.1073/pnas.0501844102

Wicher D, Schäfer R, Bauernfeind R, Stensmyr MC, Heller R Heinemann SH, Hansson BS (2008). *Drosophila* odorant receptors are both ligand-gated and cyclic nucleotide-activated cation channels. Nature 452: 1007–1012.

Willott E, Tran HQ (2002) Zinc and *Manduca sexta* hemocyte functions. Journal of Insect Sciences. 2: 9.

Wilke ABB, Christe R de O, Multini LC, et al (2016) Morphometric wing characters as tool for Mosquito Identification. PLOS ONE 11: e0161643. doi: 10.1371/journal.pone.0161643

Woestmann L, Saastamoinen M (2016) The importance of trans-generational effects in Lepidoptera. Current Zoology 62:489–499. doi: 10.1093/cz/zow029

Wolf JB, Wade MJ (2009) What are maternal effects (and what are they not)? Philosophical Transactions of the Royal Society B: Biological Sciences 364:1107–1115. doi: 10.1098/rstb.2008.0238

Wong J, Morrison AC, Stoddard ST, et al (2012) Linking oviposition site choice to offspring fitness in *Aedes aegypti*: Consequences for targeted larval control of dengue vectors. PLoS Neglected Tropical Diseases 6: e1632. doi: 10.1371/journal.pntd.0001632

Wootton RJ, Herbert RC, Young PG, Evans KE (2003) Approaches to the structural modelling of insect wings. Philosophical Transactions of the Royal Society B: Biological Sciences 358:1577–1587. doi: 10.1098/rstb.2003.1351

Wootton RJ. (1981) Support and deformability in insect wings. Journal of Zoology.; 193:447–68.

Wondwosen B, Birgersson G, Seyoum E, et al (2016) Rice volatiles lure gravid malaria mosquitoes, *Anopheles arabiensis*. Scientific Reports 6: doi: 10.1038/srep37930

Whitman DW, Agrawal A (2009) What is phenotypic plasticity and why is it important? In Whitman DW Ananthakrishnan TN (Eds.), Phenotypic plasticity of insects. Science Publishers. 1–63 pp.

Yang C -h., Belawat P, Hafen E, et al (2008) Drosophila egg-laying site selection as a system to study simple decision-making processes. Science 319:1679–1683. doi: 10.1126/science.1151842

Yang S-T, Shiao S-F (2012) Oviposition preferences of two forensically important blow fly species, *Chrysomya megacephala* and *C. rufifacies* (Diptera: Calliphoridae), and implications for Postmortem Interval Estimation. Journal of Medical Entomology 49:424–435. doi: 10.1603/ME11133

Yeap H, Axford JK, Popovici J, et al (2014a) Assessing quality of life-shortening Wolbachia-infected *Aedes aegypti* mosquitoes in the field based on capture rates and morphometric assessments. Parasites & Vectors 7:58. doi: 10.1186/1756-3305-7-58

Yeap HL, Hoffmann AA, Ross PA, Endersby NM (2014b) Larval competition extends developmental time and decreases adult size of wMelPop Wolbachia-Infected *Aedes aegypti.* The American Journal of Tropical Medicine and Hygiene 91:198–205. doi: 10.4269/ajtmh.13-0576

Yeruhan I, Braverman Y (1995) Skin lesions in dogs, horses, and calves caused by the stable fly *Stomoxys calcitrans* (L.) (Diptera: Muscidae). *Revue de médecine vétérinaire des pays tropicaux*. 4: 347-349.

Yoshioka M, Couret J, Kim F, et al (2012) Diet and density dependent competition affect larval performance and oviposition site selection in the mosquito species *Aedes albopictus* (Diptera: Culicidae). Parasites & Vectors 5:225. doi: 10.1186/1756-3305-5-225

Zhang H-J, Faucher CP, Anderson A, et al (2013) Comparisons of contact chemoreception and food acceptance by larvae of polyphagous *Helicoverpa armigera* and oligophagous *Bombyx mori*. Journal of Chemical Ecology 39:1070–1080. doi: 10.1007/s10886-013-0303-2

Zhu JJ, Zhang Q, Taylor DB, Friesen KA (2016) Visual and olfactory enhancement of stable fly trapping: Visual and olfactory enhancement of stable fly trapping. Pest Management Science 72:1765–1771. doi: 10.1002/ps.4207

Zumpt F (1973) The Stomoxyine biting flies of the world. Taxonomy, biology, economic importance and control measures. Gustav Fischer Verlag Editor, Stuttgart, 175 pages

Zwarts L, Vanden Broeck L, Cappuyns E, et al (2015) The genetic basis of natural variation in mushroom body size in *Drosophila melanogaster*. Nature Communications 6: doi: 10.1038/ncomms10115.

Appendix

SCIENTIFIC REPORTS

OPEN

Egg-laying decisions based on olfactory cues enhance offspring fitness in *Stomoxys calcitrans* L. (Diptera: Muscidae)

Received: 8 November 2018
Accepted: 11 February 2019
Published online: 07 March 2019

Steve B. S. Baleba[1,2], Baldwyn Torto[1,2], Daniel Masiga[1], Christopher W. Weldon[2] & Merid N. Getahun[1]

Selection of oviposition substrate is critical in holometabolous insects. Female stable flies, *Stomoxys calcitrans*, locate and select vertebrate herbivore dung in which they lay their eggs. However, the preference for vertebrate herbivore dung by *S. calcitrans* females, its fitness consequences for offspring, and the semiochemicals used to locate and select oviposition substrates remain unclear. Using oviposition choice tests and life table bioassays we found that gravid female *S. calcitrans* prefer to oviposit on donkey and sheep dung, which also improves the performance of their offspring. GC-MS analysis followed by random forest classification identified β-citronellene and carvone as the most important predictive volatile organic compounds of donkey and sheep dung, respectively. In multiple choice oviposition bioassays, *S. calcitrans* laid more eggs in wet sand containing β-citronellene and carvone than in other treatments. The attractiveness of these compounds was confirmed in a field trial, with traps baited with β-citronellene and carvone catching more *S. calcitrans*. We conclude that gravid female *S. calcitrans* use semiochemical cues to choose oviposition substrates that maximise offspring fitness.

In holometabolous insects, oviposition site selection by gravid females plays an important role in their distribution, abundance, and population dynamics owing to the immobility of egg stages combined with the lack of parental care[1]. Location and selection of the most appropriate substrate for oviposition involves visual, olfactory and mechanical cues[2]. According to Städler[3], the nutritional and chemical composition of the environment determine the success of development in almost all insects, thus olfactory cues play a paramount role during oviposition site selection. When selecting a suitable site for oviposition, insects might use a single chemical cue[4,5] or a mixture of important chemical cues[6–9].

Jaenike[10] postulated that gravid female insects prefer to oviposit on substrate that maximise the fitness of their offspring. This was termed the 'preference-performance' or 'mother knows best' hypothesis. Oviposition substrates used by female insects include animal dung, fruits, and leaves which are abundant and rich in nutrients[4–6]. The preference-performance relationship has been widely explored in phytophagous insects, with some studies confirming a positive relationship between preference and performance[7–9], while others show poor correspondence[11–15]. In hematophagous insects, understanding of the preference-performance relationship is most advanced in mosquitoes. For instance, there is a positive correlation between oviposition preference and larval performance in *Culiseta longiareolata*[16], *Aedes triseriatus*, and *Aedes albopictus*[17]. whereas in *Wyeomyia smithii*[18] and *Aedes aegypti*[19], a negative correlation has been observed. However, these studies do not consider the chemical basis driving the preference-performance interaction. Here we investigated the preference-performance hypothesis in the stable fly, *Stomoxys calcitrans* (Diptera: Muscidae), and the chemical basis involved in this interaction.

Stomoxys calcitrans is a cosmopolitan blood-feeding insect of medical and veterinary importance[20]. For the success of their mating, egg production and survival, both female and male *S. calcitrans* depend on repeated blood meals from diverse domestic (e.g., camel, cattle, horse) and wild animal hosts (e.g., buffalo, antelope, zebra)[21,22] as well as humans[23]. During their blood meals, *S. calcitrans* can transmit viruses (e.g., West Nile fever virus)[24], bacteria (e.g. *Bacillus anthracis*)[25], protozoans (e.g. *Trypanosoma evansi*)[26] and helminths (e.g. *Habronema*

[1]International Centre of Insect Physiology and Ecology (icipe), P.O. Box 30772-00100, Nairobi, Kenya. [2]Department of Zoology and Entomology, University of Pretoria, Private Bag X20, Hatfield, 0028, South Africa. Correspondence and requests for materials should be addressed to M.N.G. (email: mgetahun@icipe.org)

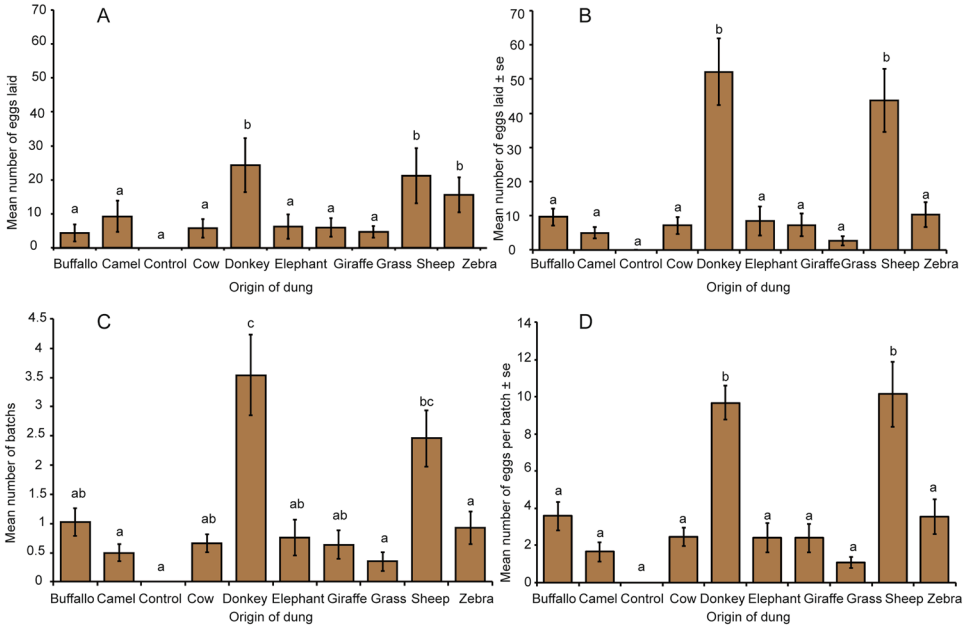

Figure 1. Gravid female *S. calcitrans* prefer to oviposit on donkey and sheep dung. (**A**) Mean number of eggs laid on dung of each vertebrate herbivore by wild female *S. calcitrans*. (**B**) Mean number of eggs laid on dung of each vertebrate herbivore by naïve female *S. calcitrans*. (**C**) Mean number of egg batches deposited on dung of each vertebrate herbivore by naïve female *S. calcitrans*. (**D**) Mean number of eggs per batch laid by naïve female *S. calcitrans*. Error bars indicate standard error of the mean (SEM). Bars with different letters are significantly different from each other (Kruskal-Wallis test followed by Dunn's post hoc test; $P < 0.05$, n = 10).

microstoma)[27]. In the USA alone, Taylor *et al.*[28] estimated that economic losses attributed to *S. calcitrans* infestation were >$2 billion per year.

The dung of vertebrate herbivore animals is used as an oviposition substrate by female *S. calcitrans*[29–31]. However, preference by *S. calcitrans* for vertebrate herbivore dung of different species, the fitness costs to its offspring, and the semiochemicals involved remain unclear. In this study, we investigated the preference-performance hypothesis in *S. calcitrans* oviposition behaviour on dung of different vertebrate herbivores, and the semiochemical basis of this interaction. We demonstrate that gravid female stable flies oviposit on substrates that have fitness benefits for their offspring using finely tuned responses to semiochemical cues in preferred substrates such as β- citronellene and carvone. We then used these semiochemicals to perform laboratory and field trials to test their attractiveness to female *S. calcitrans*. This information will prove useful for developing effective lures to attract and kill gravid females, and thereby suppress *S. calcitrans* abundance.

Results

Gravid female *S. calcitrans* prefer donkey and sheep dung for oviposition. Gravid female *S. calcitrans* consistently chose donkey and sheep dung as oviposition substrates over the other dung tested. The mean number of eggs laid on each substrate by gravid females was significantly different (Fig. 1A, Kruskal-Wallis test: H = 30.702, d.f = 9, $P < 0.001$). Females laid more eggs on donkey and sheep dung followed by zebra dung than on the dung of cow, camel, buffalo, elephant, or giraffe, grass and wet sand (control). Results obtained in the laboratory with naïve gravid female flies (7–10 days old) mirrored the results obtained in the semi-field assays (Fig. 1B; H = 59.497, d.f = 9, $P < 0.001$). The higher number of eggs laid on donkey and sheep dung resulted from a higher mean number of batches (Fig. 1C; H = 54.13, d.f = 9, $P < 0.001$) and mean number of eggs per batch (Fig. 1D; H = 59.38, d.f = 9, $P < 0.001$).

Preferred oviposition substrates enhance fitness of *S. calcitrans* offspring. Having shown that gravid female *S. calcitrans* prefer to oviposit in the dung of particular vertebrate herbivores, we tested whether female preference led to improved performance by offspring. Egg hatchability, larval and pupal development time, larval weight and pupal weight were recorded as fitness parameters. We found that egg hatchability (GLM, $\chi^2 = 18.355$, d.f = 3, $P < 0.001$), larval development time ($F_{3,36} = 132.2$, $P < 0.001$), larval weight (Day 5: H = 130.52, d.f = 3, $P < 0.001$; Day 10: H = 147.26, d.f = 3, $P < 0.001$), pupal development time or adult emergence ($F_{3,36} = 17.48$, $P < 0.001$), larval growth rate (H = 136.41, d.f = 3, $P < 0.001$), pupal weight ($F_{3,156} = 140.4$, $P < 0.001$) and adult emergence time ($F_{3,36} = 17.48$, $P < 0.001$) were affected by dung type. Egg hatchability was highest in donkey followed by camel, cow and sheep dung (Fig. 2A). Development time from egg to pupal stage was significantly shorter in donkey and sheep dung than in cow and camel dung (Fig. 2B). Larvae that developed in donkey and sheep dung were heavier at days 5 and 10 (Fig. 2D); consequently, they had the best growth rates (Fig. 2E). Pupae from donkey and sheep dung weighed more than those from cow and camel dung (Fig. 2F).

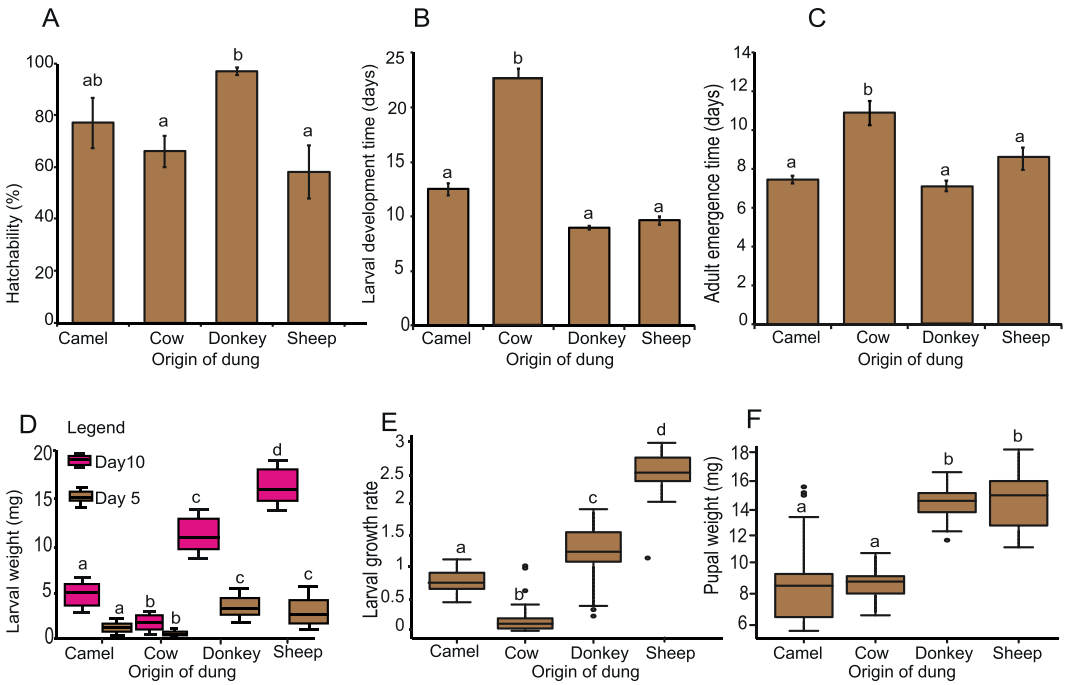

Figure 2. Gravid female *S. calcitrans* prefer to oviposit on substrates that enhance the fitness of offspring. (**A–C**) Bar plots showing: (**A**) egg hatchability, (**B**) larval development time, (**C**) adult emergence times when *S. calcitrans* offspring were reared in camel, cow, donkey, and sheep dung. Error bars represent SEM. Bars with different letters are significantly different from each other (ANOVA followed by SNK post hoc test; $P < 0.05$, $n = 10$). (**D–F**) Boxplots illustrating: (**D**) larval weight at five (brown) and ten days (pink), (**E**) larval growth rate, (**F**) pupal weight of *S. calcitrans* when raised in camel, cow, donkey, and sheep dung. Boxplot whiskers indicate ± 1.5 interquartile range limits. Box plots with different letters are significantly different from each other [grouped by the Kruskal-Wallis test followed by Dunn's post hoc test for larval weight and larval growth rate, and ANOVA followed by SNK's post hoc test for the pupal weight ($P < 0.05$, $n = 10$)].

Preference-performance relationship positively correlated with the physicochemical composition of preferred substrates. Our results clearly demonstrated that oviposition preference of gravid female *S. calcitrans* contributes to fitness of their offspring. Due to this, we set out to determine whether the preference-performance relationship was correlated with the physicochemical composition of the different oviposition substrates. We analysed the physical properties and micronutrient contents of the two preferred substrates (donkey and sheep dung) and non-preferred substrates (camel and cow dung). There was a significant difference in the physiochemical properties of the different animal dung (Fig. 3A, MANOVA: Pillai's trace = 2.99, $F_{3,36} = 2734$, $P < 0.001$). Cow dung had the highest percentage of water content (Fig. 3A.1) and the lowest percentage of dry matter (Fig. 3A.2). The pH was lowest in donkey dung (Fig. 3A.3). We found a lower carbon/nitrogen ratio (Fig. 3A.4) and a higher amount of copper (Fig. 3A.9) in sheep dung. Phosphorus (Fig. 3A.5), potassium (Fig. 3A.6) and zinc (Fig. 3A.8), were higher in donkey and sheep dung; while calcium was highest in camel dung (Fig. 3A.7).

To correlate oviposition choice, fitness and physicochemical parameters, we performed a principal component analysis (PCA). We found these parameters to be highly correlated (Fig. 3B), indicating that *S. calcitrans* gravid females appear to consider the physiochemical properties of substrates when deciding to oviposit. The first two dimensions of the PCA explained 81.6% of the total variation. Dimension 1 was associated with the proportion of phosphorus and potassium in dung, and accounted for 50% of the total variation. Dimension 2, which accounted for 31.6% of the total variation, was highly correlated with carbon/nitrogen ratio and the proportion of copper in dung. To clearly illustrate correlations among measured variables, we constructed a correlogram (Fig. 3C). Oviposition was positively correlated with larval weight ($r = 0.58$, $P < 0.001$) and pupal weight ($r = 0.64$, $P < 0.001$); and negatively correlated with larval developmental time ($r = -0.48$, $P < 0.001$). Larval weight and pupal weight were positively correlated with the proportion of zinc ($r = 0.59$, $P < 0.001$; $r = 0.75$, $P < 0.001$), potassium ($r = 0.59$, $P < 0.001$; $r = 0.73$, $P < 0.001$), phosphorus ($r = 0.68$, $P < 0.001$; $r = 0.77$, $P < 0.001$), and copper ($r = 0.75$, $P < 0.001$; $r = 0.42$, $P = 0.0074$) which were more abundant in preferred oviposition substrates. Larval weight and pupal weight were negatively correlated with the proportion of calcium ($r = -032$, $P = 0.0436$; $r = -0.55$, $P < 0.001$) and carbon/nitrogen ratio ($r = -0.74$, $P < 0.001$; $r = -0.42$, $P = 0.0074$), which were higher in non-preferred oviposition substrates. Larval developmental time was positively correlated with dung water content ($r = 0.66$, $P < 0.001$) and carbon/nitrogen ratio ($r = 0.73$, $P < 0.001$), and negatively correlated with the proportion of phosphorus ($r = 0.69$, $P < 0.001$), potassium ($r = 0.66$, $P < 0.001$), and copper ($r = -0.66$, $P < 0.001$), which were again higher in donkey and sheep dung.

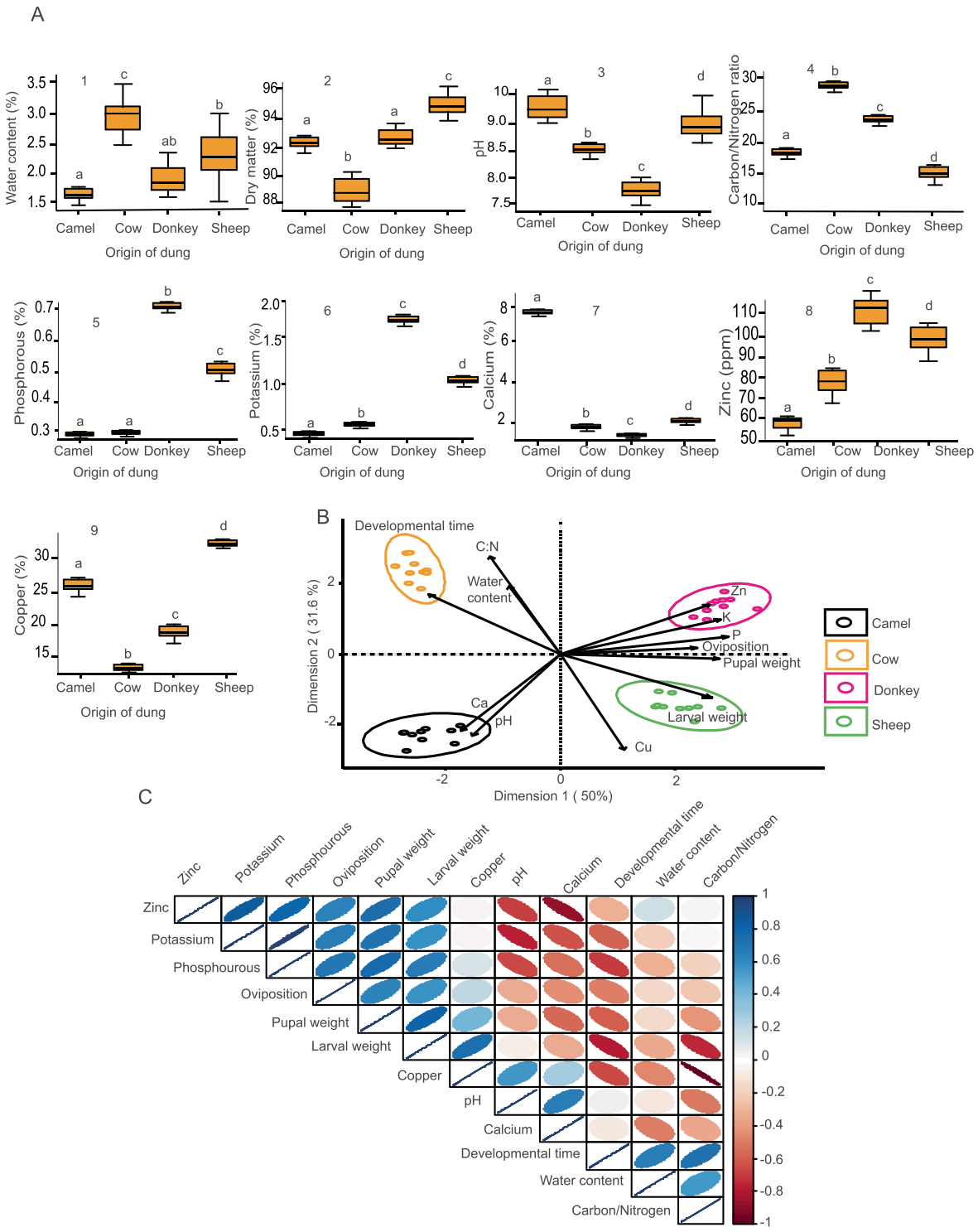

Figure 3. Oviposition substrates vary in their physiochemical properties. (**A**) Boxplot depicting results of physicochemical analysis of camel, cow, donkey and sheep dung: (1) water content, (2) dry matter, (3) pH, (4) carbon/nitrogen ratio, (5) phosphorus proportion, (6) potassium proportion, (7) calcium proportion, (8) zinc proportion and (9) copper proportion. The ends of boxplot whiskers represent the minimum and maximum of all the data. Boxes with different letters are significantly different from each other based on MANOVA followed by SNK post-hoc tests. (**B**) Principal component biplot showing the relation between *S. calcitrans* oviposition preference, larval performance and dung composition. Black ellipse: camel dung; orange ellipse: cow dung; magenta ellipse: donkey dung; and green ellipse: sheep dung. (**C**) Correlogram highlighting the direction and intensity of the correlation between oviposition preference, larval performance traits, and dung composition. Red and blue denote high negative and positive correlation, respectively; white indicates absence of correlation.

Oviposition substrates are distinct in their volatile organic compound (VOC) composition. We determined whether oviposition preference behaviour observed in gravid female *S. calcitrans* (Fig. 1) was potentially mediated by olfactory cues by using coupled gas chromatography-mass spectrometry (GC-MS) to analyse the VOC composition of all dung types used in the oviposition bioassays. GC-MS analysis identified a wide range of VOCs emitted by the substrates (Fig. 4A). Using multidimensional scaling ordination, we found that each dung type had a distinct VOC composition (Fig. 4C).

Signature VOCs of donkey and sheep dung elicit strongest oviposition. We hypothesised that gravid females of *S. calcitrans* use signature VOCs to locate suitable oviposition substrates, and that in our study these signature VOCs are represented by compounds that are abundant and permanently present (most important volatiles) in donkey and sheep dung. To test this hypothesis, we first classified the VOCs of each dung using a classification algorithm method called "random forest" (RF), which has been used successfully in others studies[32–34]. Based on the function *"importance ()"* embedded in the Random Forest R software package, we obtained the mean decrease in accuracy (MDA) of each volatile present in each dung. As the rule of thumb, VOCs with the highest MDA are, the most important, namely abundant and permanently present across all the replicates of tested vertebrate herbivore dung. Based on that rule we found that carvone was the most important VOC of buffalo and sheep dung; while *p*-cymene, limonene, β-citronellene, cyperene, *m*-cresol, and camphene were the most important VOCs of camel, cow, donkey, elephant, giraffe, and zebra dung, respectively (Fig. 4B), with a classification accuracy of 87.9%.

Having determined the most important VOC of each dung, we next asked if the most important VOC from donkey and sheep dung would stimulate more gravid female *S. calcitrans* to oviposit. To test this, we conducted a multiple-choice oviposition bioassay where gravid females of *S. calcitrans* were presented with wet sand loaded with the synthetic standard of the single most important VOC of each substrate at 10^{-2} v/v dilution. We found that oviposition by *S. calcitrans* varied across the tested VOCs (Fig. 4D: $H = 25.30$, $df = 6$, $P < 0.001$). In comparison with other media, gravid female *S. calcitrans* laid more eggs on the media loaded with β-citronellene and carvone, which are the most important VOCs of preferred oviposition substrates, donkey and sheep dung, respectively. To verify whether only β-citronellene and carvone led to the observed increase in number of eggs laid, we replaced β-citronellene and carvone with β-caryophyllene and *m*-xylene, which are the most important VOCs from donkey and sheep dung, respectively, and recorded oviposition. Neither β-caryophyllene nor *m*-xylene induced a significant increase in the mean number of eggs laid (Fig. 4E: $H = 3.78$, $df = 6$, $P = 0.71$). We concluded that β-citronellene and carvone were signature VOCs used by gravid females as olfactory cues to identify the best oviposition substrates.

β- Citronellene and carvone enhance trap catch of *S. calcitrans*. Having identified the VOCs that stimulate gravid female *S. calcitrans* to oviposit more on donkey and sheep dung, we assessed their attractiveness under field conditions. A Latin square design experiment was performed at Mpala Research Centre (www.mpala.org) located in Laikipia County, Kenya (Fig. 5A) using monoconical traps (Fig. 5B). Traps were baited with different VOCs (Fig. 5C) and rotated daily to account for any bias resulting from trap location[35]. Each treatment consisted of an undiluted solution of β-citronellene, carvone, Blend A (carvone + β-citronellene), Blend B (β-citronellene + valencene), Blend C (carvone + valencene + γ- terpinene), *m*-cresol (positive control: already known to attract *S. calcitrans*; see Tangtrakulwanich[36]), or a negative control (unbaited trap).

Overall, we caught more *S. calcitrans* [8702 (85%)] than other insects [1739 (15%)] ($\chi^2 = 4643.6$, $df = 1$, $p < 0.001$). Other insects mainly comprised house flies, *Musca domestica* [1706 (98.1%)]. The number of *S. calcitrans* caught significantly varied with VOC treatment (GLM.nb: $LR = 15.74$, $df = 6$, $P < 0.05$). The mean number of *S. calcitrans* caught by traps baited with β-citronellene, Blend A, Blend B, carvone, Blend C and *m*-cresol were respectively 3.9, 3.3, 3.30, 2.6, 2.5 and 2.4 times more than those caught in unbaited traps (Fig. 5D).

Irrespective of VOC treatment, we caught significantly more blood fed (58.6%)) than unfed *S. calcitrans* (41.41%) ($\chi^2 = 256.5$, $df = 1$, $P < 0.001$). The number of blood-fed flies varied significantly among VOC treatments (GLM.nb: $LR = 9.83$, $df = 6$, $P < 0.05$). Traps baited with β-citronellene, Blend A, Blend B, carvone and Blend C caught significantly more blood-fed *S. calcitrans* than the unbaited trap. Conversely, trap catches of blood-fed *S. calcitrans* with *m*-cresol baited and unbaited traps were not significantly different (Fig. 5E).

During the entire period of trapping, we caught significantly more female *S. calcitrans* (65%) than males (35%) ($\chi^2 = 741.39$, $df = 1$, $P < 0.001$). The VOC treatment significantly affected the sex ratio (female to total number of individuals ratio) of caught flies ($F_{6,42} = 3.74$, $P = 0.0045$). The sex-ratio was female-biased in traps baited with β-citronellene, carvone, Blend B, Blend A, and *m*-cresol, while the unbaited traps caught the same number of females and males (Fig. 5F).

The number of gravid females caught significantly differed among the VOC treatments (GLM.nb: $LR = 14.35$, $df = 6$, $P < 0.05$). Among all the VOC treatments, only traps baited with β-citronellene caught significantly more gravid females of *S. calcitrans* than the unbaited traps. The mean number of gravid flies caught by traps baited with carvone, *m*-cresol, Blend A, Blend B, Blend C was not significantly different from the unbaited trap catch (Fig. 5G).

Discussion

Our results demonstrate that gravid female *S. calcitrans* exhibit a preference-performance oviposition behaviour that is mediated by olfactory cues. Additionally, these results demonstrate that oviposition preference is associated with better fitness of *S. calcitrans* immature stages.

Results from the oviposition preference bioassay revealed that gravid female *S. calcitrans* preferred to lay eggs on donkey dung followed by sheep dung. This has also been reported by Hafez & Gamal-Eddin[30] but the causes for this preference and the fitness benefits were not investigated. We demonstrated that oviposition preference by

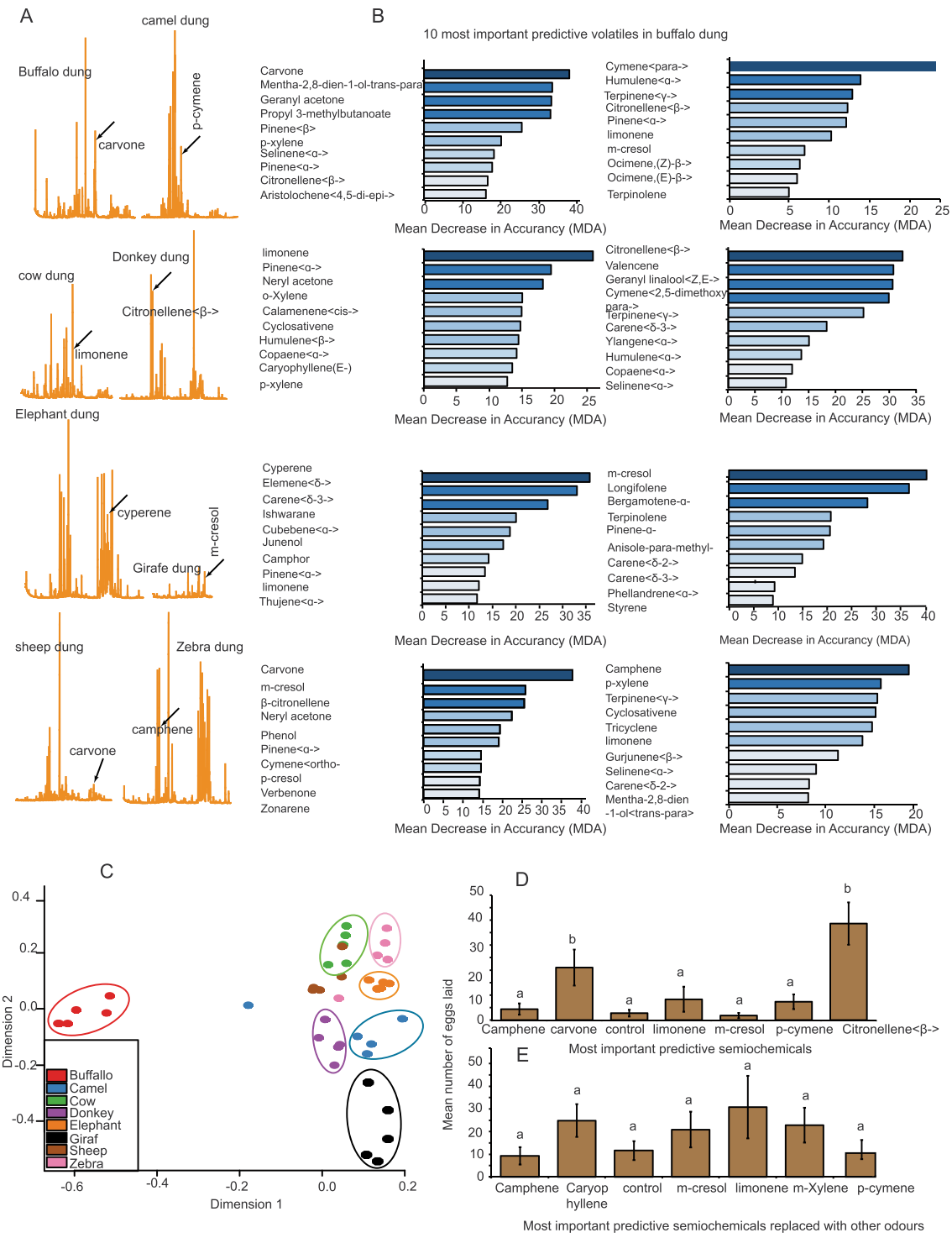

Figure 4. The most important volatiles from donkey (β-citronellene) and sheep (carvone) dung elicit the strongest oviposition response by *S. calcitrans* gravid females. (**A**) Representative GC–MS chromatogram of each vertebrate herbivore dung and most important semiochemicals. (**B**) Multidimensional scaling (MDS) plot showing the segregation of vertebrate herbivore dung based on their VOC composition. (**C**) Histogram showing the classification of the ten most important VOCs from vertebrate herbivore dung based on the Mean Decrease in Accuracy (MDA) of the Random Forest analysis. VOC associated with the darkest histogram has the highest MDA value and consequently, the most important. (**D**,**E**) Bar plots representing: (**D**) mean number of eggs laid by gravid female *S. calcitrans* on each oviposition medium (wet sand) loaded with the most important VOC of each vertebrate herbivore dung. Error bars represent SEM. (**E**) mean number of eggs laid by gravid female *S. calcitrans* on each oviposition medium (wet sand) loaded with the most important chemical volatile of each vertebrate herbivore dung with the replacement of β-citronellene and carvone by β-caryophyllene and m-xylene, respectively. Bars with different letters are significantly different from each other (Kruskal-Wallis test followed by Dunn's post hoc test; $P < 0.05$). Error bars represent SEM.

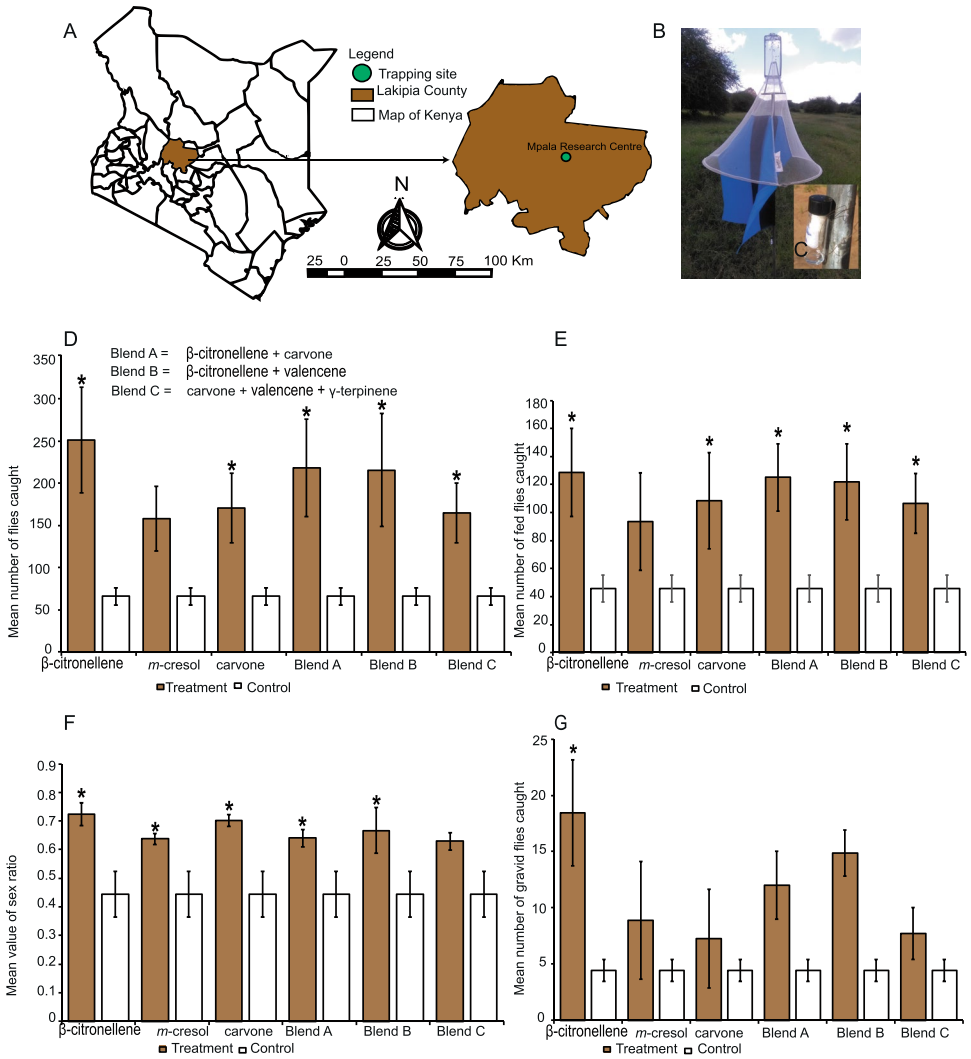

Figure 5. β-citronellene and carvone significantly enhance *S. calcitrans* trap catch. (**A**)Trapping site map, (**B**) Monoconical trap (**C**) Cotton roll dispenser in 4 ml vial with perforated cap. (**D–G**) bar plots depicting: (**D**) the mean number of *S.calcitrans* caught, (**E**) the mean number of *S.calcitrans* blood fed flies caught, (**F**) the mean value of *S.calcitrans* sex ratio, (**G**) the mean number of *S.calcitrans* gravid females caught. Error bars represent SEM., treatment with an asterisk (*) above the error bar are significantly different from the negative control (unbaited trap) [Dunnett's t-test ($P < 0.05$, $n = 7$)].

female *S. calcitrans* was related to better fitness of *S. calcitrans* immature stages. For instance, larvae and pupae of *S. calcitrans* were heavier and developed more rapidly on donkey and sheep dung. This may reflect the higher nutritional value of these specific substrates for *S. calcitrans*[37]. We show that nitrogen, zinc, potassium, phosphorous and copper content were significantly higher in donkey and sheep dung. These elements were positively correlated with larval and pupal weights and negatively correlated with developmental time. The positive effect of these elements in insect fitness is widely acknowledged in the literature[38–41]. Similarly, Perkins *et al.*[42] demonstrated that augmentation of the diet with phosphorus increased the growth rate of the tobacco hornworm, *Manduca sexta* (L) (Lepidoptera: Sphingidae).

Evidence for the preference-performance relationship has been demonstrated in other insect groups although studies have been heavily biased towards herbivorous insects. For example, Heisswolf *et al.*[43] found that females of the monophagous beetle *Cassida canaliculata* (Coleoptera: Chrysomelidae) preferred to oviposit on larger host plants (rich in nitrogen), which then led to improved larval performance and survival in comparison with larvae developing on smaller host plants. Similarly, Chen *et al.*[44] have shown that cotton with a higher level of nitrogen is suitable for oviposition and development of larvae of the beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae). When offered a choice between two cultivars of *Brassica oleracea* (Derby Day and Drago), female diamondback moths, *Plutella xylostella* (Lepidoptera: Plutellidae) laid more eggs on the cultivar with lower glucosinolate concentration, which also maximised larval performance[45].

Our results revealed that the preference-performance behaviour observed in *S. calcitrans* was driven by olfactory cues. We demonstrated that although vertebrate herbivore dung contained a plethora of VOCs [more than

45 each (Fig. 4A)], *S. calcitrans* used a single semiochemical to select an oviposition site. For instance, only one VOC identified using random forest analysis was enough to mimic the presence of preferred or unpreferred vertebrate herbivore dung. As predicted, the most important VOC from donkey dung (β-citronellene) was enough to mimic the stimulatory effect of the substrate. On the other hand, media loaded with carvone did not significantly differ from the other media (Fig. 5D). This result shows that to mimic the response of gravid *S. calcitrans* to sheep dung, some components might be missing. Several studies report that a single compound might be enough for females to detect oviposition substrates and initiate oviposition[4,46], while in other cases a signature blend of VOCs might be required for the same outcome[6,47]. In the absence of β-citronellene and carvone, *S. calcitrans* gravid females failed to exhibit a preference for any substrate. Instead, they laid eggs randomly on each presented medium including those that led to poor fitness, demonstrating the importance of β-citronellene and carvone in oviposition-site selection. However, it is not clear how these semiochemicals relate to the nutrient value of the substrates. It is likely that other sensory modalities play a role, such as taste. Jeanbourquin & Guerin[29] reported the presence of β-citronellene in horse dung (like donkey, in the family Equidae) with electrophysiological activity in *S. calcitrans* antennae, but the authors did not go on to explore the effect of this chemical on *S. calcitrans* behaviour.

Synthetic semiochemicals eliciting a specific behaviour in insects can enhance insect catches when used as a bait in field trapping systems[48–50]. This was confirmed in our field work when we baited monoconical traps with β-citronellene, carvone, Blend A, Blend B, and Blend C. In this experiment, *S. calcitrans* represented 85% of insects caught, which supports the results of Mihok *et al.*[51] and Tunnakundacha *et al.*[52] who reported that monoconical traps are efficient for trapping flies in the subfamily Stomoxyinae. Traps baited with β-citronellene, carvone, Blend A, Blend B, and Blend C captured more flies than unbaited traps (negative control) and traps baited with *m*-cresol (positive control) (Fig. 5D). When checked for feeding status, traps baited with the same VOC treatments captured more engorged flies than the unbaited traps (Fig. 5E). A possible explanation for this result is that vertebrate herbivore dung is more likely to be visited by fed females for oviposition. Additionally, males of *S. calcitrans* are also attracted to dung-derived semiochemicals, most probably to look for females for mating. Interestingly, when we checked for gravid status, we found that only traps baited with β-citronellene caught significantly (4.5 times) more gravid female *S. calcitrans* than unbaited traps. Also, traps baited with blends containing β-citronellene [Blend A (carvone + β-citronellene) and Blend B (β-citronellene + valencene)] captured more gravid flies than traps baited with carvone, *m*-cresol, and Blend C (carvone + valencene + γ-terpinene) (Fig. 5G). This clearly shows that, either as a single or combined with other dung volatiles, β-citronellene efficiently attracts gravid female *S. calcitrans*.

In conclusion, gravid female *S. calcitrans* exhibit a preference-performance oviposition behaviour driven by signature odours emanating from vertebrate herbivore dung. Larvae and pupae developing from eggs laid on preferred substrates exhibit higher fitness than those that develop on non-preferred substrates. Furthermore, a single or blend of signature VOCs such as β-citronellene and carvone increased *S. calcitrans* female attraction and stimulated oviposition both under laboratory and field conditions. The high level of female-biased attraction, including high levels of gravid female and blood-fed flies, to the semiochemical β–citronellene associated with oviposition substrates, is promising for its potential use in the management of *S.calcitrans*.

Material and Methods

Insects and oviposition substrates. To establish the colony, wild individuals of *S. calcitrans* were captured at *icipe* Duduville campus in Nairobi (1°13′12″S, 36°52′48″E; 1,600 m above sea level) using a Vavoua trap. Trapped adults were transferred to cages (75 × 60 × 45 cm) in an insectary maintained at 25 ± 5 °C and 65 ± 5% relative humidity, with a 12 L: 12 D photocycle. Flies were fed two times per day (0800 and 1600 hours) on defibrinated bovine blood on moistened cotton. The rearing medium consisted of rabbit dung. Rabbit dung was placed in plastic containers (21.5 × 14.5 × 7.4 cm) that were introduced to the adult cage for two days to allow oviposition. Afterwards, the rearing medium was removed and transferred to another cage and followed daily from egg hatch to the pupal stage. Pupae were placed in Petri dishes, removed from the cage, and introduced to another cage for age-matched adult emergence. As described above, blood and rearing media were provided to the newly emerged adults to obtain flies for experiments.

For our oviposition bioassay, we screened fresh dung of buffalo, camel, cow, donkey, elephant, giraffe, sheep, and zebra, which are potential breeding sites for *S. calcitrans* and abundant in the region where the study was conducted. The dung types were collected from different agroecological zones, including Kapiti Plain in Machiakos County (1°37′60″S, 37°0′0″E), Ngurunit in Marsabit County (1°59′58″S, 37°30′11″E), and Shimba Hills located in Kwale County (04°15′26″S, 39°23′16″E), Kenya. These localities are characterised by the presence of several populations of wild and domestic vertebrate herbivores, as well as biting flies such as *S. calcitrans*. Dung was collected immediately after it was deposited by each species (within 24 hours). To our knowledge, none of the vertebrate herbivore populations from which dung was collected received anthelmintic treatments that could potentially affect development of coprophagous insects[53].

Preference-performance hypothesis test. *Multiple-choice oviposition preference bioassay with vertebrate herbivore dung.* To assess oviposition preference of gravid female *S. calcitrans*, we began with a multiple-choice oviposition bioassay in semi-field conditions, using wild, gravid female, easily recognized by examining the ventral abdomen filled with eggs. *S. calcitrans* caught directly from the field. A cage (75 × 60 × 45 cm) containing 30 wild gravid females was placed outside in a shaded, sheltered location. Fresh dung (60 g) from buffalo, camel, cow, donkey, elephant, giraffe, sheep, and zebra, as well as grass and wet sand (control), was placed in Petri dishes and introduced to the cage. Each Petri dish was separated by at least 20 cm in a circle, with the order of dung being haphazard. Twenty-four hours after setting up the bioassay, the total number of eggs laid on each substrate was counted. The cages were placed outside at the *icipe* Nairobi campus from May to June 2017, during which 30

replicates were performed. Mean temperature during this period was 22.5 ± 4.7 °C, with 55% relative humidity, and 12 L: 12 D photoperiod.

To check the validity of the result from field collected flies under semi-field conditions, we conducted the same experiment in a controlled laboratory environment (at 25 ± 5 °C and 65 ± 5% relative humidity, with a 12 L:12 D photocycle) using 30 naïve, gravid females *S. calcitrans* (aged from 4 to 6 days) from our established culture. Here, the oviposition preference was assessed after 24 hours by recording three parameters: (1) number of batches deposited on each substrate, (2) number of eggs per batch, and (3) the total number of eggs deposited on each substrate. The laboratory experiment was replicated ten times.

Performance test. We conducted an incomplete cohort life table study for the immature stages of *S. calcitrans* using camel, cow, donkey and sheep dung. This was to test if a detected preference of gravid female *S. calcitrans* to oviposit on donkey and sheep dung in comparison with camel and cow dung was related to the performance of their offspring. Ten *S. calcitrans* eggs were artificially introduced to each type of dung (n = 10) and followed daily until adulthood to record six parameters: (1) egg hatchability, (2) larval development time (from egg to pupa), (3) pupal development time (from pupa to adult eclosion), (4) larval weight, (5) larval growth rate and (6) pupal weight. Eggs for each treatment were obtained by placing the same dung type in the established culture cage 24 hours before commencing the test. The weight parameter was recorded individually on 40 larvae and pupae coming from each substrate. Larval weight was recorded 5 and 10 days after egg hatch. Larval growth rate was calculated as[13] $[M_{day10} - M_{day5}]/t$, where M_{day10} was the larval mass at day ten, and M_{day5} was the larval mass at day five, and t, was the number of days intervening between the two consecutive weight measurements.

Dung sample physico-chemical characterisation. To elucidate whether the performance of *S. cacitrans* offspring is related to the chemical composition of their substrate of development, we determined the proportion of nitrogen (N), carbon (C), pH, and micronutrients [copper (Cu), phosphorus (P), potassium (K), zinc (Zn)] present in camel, cow, donkey and sheep dung using lyophilised (freeze-dried), ground samples (n = 10). The total N was determined using the Kjeldahl method with 0.5 g of each dung material[54]. The total C expressed as a percentage of residues was determined after 4 hours of ignition at 500 °C in a muffle furnace using a 0.5 g sample of each dung[55]. The pH was measured using the potentiometric method after water sample extraction (by shaking 1:2 w/v of each sample for 20 minutes at 180 rpm). The micronutrients were measured from 0.5 g of each sample using the atomic emission spectrometry (ICP-OES) method following the microwave digestion procedure with nitric acid and hydrochloric acid[56]. Additionally, to determine the water content of each animal dung, we recorded the volume (V) and weight (W_{wet}) of ten fresh samples. Afterwards, we determined the dry weight (W_{dry}) of the same samples by placing them in an oven at 100 °C and reweighing them several times until constant weight. We calculated the water content, and the proportion of dried matter of each animal dung using the formula[54] $(W_{wet} - W_{dry})/ W_{dry}$; and $(W_{wet} - W_{dry})/V$.

Characterisation of semiochemicals emanating from oviposition substrates. *Odour collection.* Odours were collected from fresh dung samples from buffalo, camel, cow, donkey, elephant, giraffe, sheep, and zebra using a dynamic headspace apparatus[57]. In this setup, ambient air was passed through copper tubing to activated charcoal (for purification), then into a bubble humidifier containing double distilled water. The humidified air was supplied by a vacuum at a flow rate of 150 ml air min^{-1} through multiple ports (manifolds) to the dung samples enclosed in glass jars, which were connected in parallel. Each glass jar had a port for a Super Q adsorbent, which trapped any volatile organic compounds from the vacuum air stream. For our collection, 500 g of each dung type (replicated five times) was introduced and sealed inside the glass jars (oven sterilised at 100 °C for 24 hours). Before use, each adsorbent was cleaned ten times with 200 µL hexane, followed by dichloromethane to avoid contamination. Volatiles were collected for 24 hours. Trapped volatile compounds were eluted by washing the adsorbent with 600 µl hexane and stored in air-tight glass vials at −20 °C until analysed by gas chromatography linked with mass spectrometry.

Gas chromatography linked with mass spectrometry. The collected volatile compounds were analysed using a gas chromatograph coupled to a mass spectrometer (GC–MS; HP 6890 GC and 5975 MS; Agilent Technologies, Palo Alto, CA, USA) in the electron impact ionisation mode at 70 eV. Each sample (1 µL) was injected into the GC-MS with an autosampler (Agilent Technologies). Injections of the volatile extracts were conducted with a splitless injector at 220 °C. Compounds were separated on a nonpolar capillary HP column with helium as the carrier gas at an average linear flow rate of 35 cm s^{-1}. The oven temperature was held at 35 °C for 5 min and then increased by 10 °C/min to a final temperature of 280 °C, which was held for 10 min. Volatiles were then identified by comparison of their mass spectra and retention times with those of commercial standards and library database spectra using the NIST mass spectral search program (ver. 2.0), Pherobase (http://www.pherobase.com) and the NIST web book (http://webbook.nist.gov/chemistry). The identified chemicals were confirmed by co-injection with authentic standards and comparison with both the expected retention time and MS spectra.

Volatile chemical classification. To identify the volatiles that are abundant and permanently present across all the replicates of each herbivorous vertebrate dung (most important volatiles), we performed random forest (RF) analysis using the relative abundance of each identified volatile organic compound[58] The RF analysis is a mathematical algorithm that uses results from several decisions trees to classify a large number of variables (chemical volatiles in our case)[59]. Compared to other classification methods such as principal component analysis (PCA), the RF analysis is advised for volatile importance classification. This is because (1) it allows for more variables; (2) it has a good classification efficiency; (3) it is capable of arriving at a minimal set of variables that can be used as predictors for a particular group; (4) it is robust to interactions and correlations among variables; (5) it

gives measures of relative variable importance; and (6) it can also be used to analyse time series data that record patterns in volatile emissions over time[59–61]

Multiple-choice oviposition bioassay with the most important dung volatiles. The RF analysis identified carvone as the most important volatile of buffalo and sheep dung. On the other hand, *p*-cymene, limonene, β-citronellene, cyperene, *m*-cresol, and camphene were identified as the most important volatiles of camel, cow, donkey, elephant, giraffe, and zebra dung, respectively. Subsequently, we purchased the synthetic standards of the following compounds: (R)-(−)-carvone (98%), *p*-cymene (99%), (R)-(+)-limonene (93%), (+)-β-citronellene (analytical standard), *m*-cresol (98%), and camphene (95%) (Sigma-Aldrich Germany). Cyperene was not tested due to its unavailability of a commercial product.

The attractiveness of each volatile for *S. calcitrans* oviposition was tested using the concentration 10^{-2} v/v diluted in mineral oil. For the bioassay, seven Petri dishes containing wet sand as an oviposition medium were introduced to a cage (75 × 60 × 45 cm). We placed an Eppendorf tube lid loaded with 100 µl of each volatile solution on the wet sand. The control was wet sand with mineral oil. Thereafter, 30 gravid females of *S. calcitrans* were introduced to the cage. After 24 hours the total number of eggs deposited on each medium was counted. The experiment was replicated 15 times. Wet sand (control) was avoided by gravid females of *S. calcitrans* in our earlier oviposition preference bioassays, so any enhancement of oviposition indicated a stimulant effect on females.

The results of this bioassay revealed that media containing carvone and β-citronellene from sheep and donkey dung, respectively, attracted more gravid females of *S. calcitrans* for oviposition over the other volatiles. To appreciate the attractiveness effect of carvone and β-citronellene in gravid female *S. calcitrans* oviposition, we conducted another oviposition preference bioassay by replacing these two volatiles with β-caryophyllene and *m*-xylene. β-caryophyllene and *m*-xylene were present in donkey and sheep dung respectively but less important. The same methods were used for this bioassay, which was replicated 15 times, and the total number of eggs laid in each Petri dish of wet sand was counted after 24 hours.

Field trapping assay. Finally, we tested the attractiveness of carvone and β-citronellene to *S. calcitrans* (mainly gravid females) under field conditions. To do so, we conducted a field trapping study at Mpala Ranch located in Laikipia County in central Kenya (Fig. 5A; 00° 23′26.98″N, 036°52′14.98″E). This region is characterised by semi-arid savannah vegetation in which *S. calcitrans* are associated with wild animals (elephant, zebra, impala, monkey, lion etc.) and domestic animals (mainly camel, cattle, goat and sheep). Vavoua traps[21]; (Fig. 5B) were baited with 2 ml of undiluted synthetic standards of β-citronellene, carvone, and *m*-cresol, which was a positive control, already known to attract *S. calcitrans*[36]. Blends of volatile chemicals were also tested. These were formulated using the mean decrease accuracy (MDA) value of their chemicals. Blend A comprised carvone +β-citronellene (3:2), Blend B comprised β-citronellene + valencene (1:1), and Blend C comprised carvone + valencene + γ-terpinene (3.5:3.5:3). Each compound or blend was transferred to a 4 ml glass vial. A cotton dental roll (10 × 38 mm; Shanghai Dochem Industries Co. Ltd.) was inserted inside the vial as a dispenser. The vial was closed with a perforated cap (5 holes of 2 mm diameter[62]) and gently tilted to soak the cotton dispenser (Fig. 5C). Traps with vials without chemicals were used as a negative control. Each vial was fixed on the pole of each trap with a metal wire, 0.5 m above the ground.

Traps were placed 150 m apart in a Latin square design. Trapping was carried out for 7 days with daily rotation of vials among the traps. Seven traps per lure were set before 09:00 h daily and checked twice per day at 13:00 h and 17:00 h. *Stomoxys. calcitrans* were identified based on the key of Zumpt[63]. Identified individuals were sorted by sex, blood-feeding status and egg development. Feeding status and egg development were established by gently piercing the abdomen of each captured female with a needle to verify the presence of blood and eggs, respectively. Three parameters were therefore recorded for each trap: (1) the number of males and females of *S. calcitrans* caught, (2) the number of fed flies caught and (3) the number of gravid females caught.

Data analysis. All analyses were performed using R software[64] (version 3.5.1) and the R Studio graphical user interface (version 1.1.383). The data from the parameters we used to assess oviposition preference of gravid female *S. calcitrans* (number of batches deposited on each substrate, number of eggs per batch and the total number of eggs laid on each substrate) were subjected to the Shapiro-Wilk test of normality and Levene's test of homoscedasticity. Data were not normally distributed, and variances were not homogeneous ($p < 0.05$). Therefore, we used the non-parametric Kruskal-Wallis test followed by Dunn's post hoc test with Bonferroni's adjustment (to avoid type I error) to determine whether *S. calcitrans* oviposition differed among the tested substrates[65].

Immature performance parameters were analysed in relation to substrate as the dependent variable. Egg hatchability data were analysed using a generalised linear model (GLM) with binomial distribution due to the binary nature of this parameter (hatched vs unhatched)[66]. The number of hatched and unhatched eggs were taken as the response variable. Model significance was detected by analysis of deviance (with the chi-squared test). Tukey's multiple comparisons tests were performed using the package 'lsmeans'[67] to identify differences in egg hatchability among substrates. Larval development and adult emergence time data were normally distributed (Shapiro-Wilk test: $p > 0.05$). so we used analysis of variance (ANOVA) followed by Student-Neuman-Keuls (SNK) post hoc multiple comparisons tests using the R software package called 'Agricolae'[68] to separate means from each substrate. Larval weight and larval growth rate data were analysed using the non-parametric Kruskal-Wallis test followed by post-hoc Dunn's tests due to the non-normal distribution of the data and the disparity of their variance. For pupal weight, data were normally distributed, so we used ANOVA followed by the SNK post-hoc tests.

To compare the physicochemical composition of camel, cow, donkey, and sheep dung, we performed the multivariate analysis of variance (MANOVA) followed by SNK tests. To establish whether dung constituents were

correlated to *S. calcitrans* oviposition preference and the performance of their offspring, we performed principal components analysis (PCA) using two R packages called "FactoMineR" and "Factoextra"[69].

We classified the chemical volatiles arising from the dung of each vertebrate herbivore, using the R software package "RandomForest"[58], version 4.6–12. To execute the RF analysis, we ran 10000 iterations (ntree) with 11 volatiles randomly selected at each split (mtry = $\sqrt{q}$, where q is the total number of volatiles (120)). Based on the function '*importance ()*" we generated the mean decrease in accuracy (MDA), which provides an importance score for each volatile. For each herbivore dung, the volatile with the highest MDA value was considered the most important[32,61]. To visualise the similarity of herbivore dung volatile composition, we generated a multidimensional scaling (MDS) ordination plot[70] using the function "*MDSplot()*"of the "RandomForest" package.

Data from multiple-choice oviposition with important volatiles were analysed with the non-parametric Kruskal –Wallis test followed by the post hoc Dunn's test.

For the results from the field trapping assay, we used a generalised linear model with negative binomial error distribution and log-link to determine whether bait enhanced *S. calcitrans* catches (package 'MASS'[71], function 'glm.nb'). Post-hoc Dunnett's tests (R function glht from the package multcomp) were used to compared the number of flies caught by each baited trap with the unbaited control trap[72]. We compared the following parameters: (1) the number of flies, (2) the number of fed flies, and (3) the number of gravid females caught by each trap. For the sex ratio parameter (female number/total number of flies), we used ANOVA followed by Dunnett's test. All statistical results were considered significant when $P < 0.05$.

Data Availability

The raw data generated and analysed in the current study are available from the corresponding author on reasonable request.

References

1. Deas, J. B. & Hunter, M. S. Mothers modify eggs into shields to protect offspring from parasitism. *Proceedings of the Royal Society B: Biological Sciences* **279**, 847–853 (2012).
2. Bentley, M. D. & Day, J. F. Chemical ecology and behavioral aspects of mosquito oviposition. *Annual review of entomology* **34**, 401–421 (1989).
3. Städler, E. Oviposition behavior of insects influenced by chemoreceptors. in *Olfaction andTaste XI* (eds Kurihara, K., Suzuki, N. & Ogawa, H.) 821–826 (Springer Japan)., https://doi.org/10.1007/978-4-431-68355-1_335 (1994).
4. Dweck, H. K. M. *et al*. Olfactory preference for egg laying on citrus substrates in Drosophila. *Current Biology* **23**, 2472–2480 (2013).
5. Guidobaldi, F. & Guerenstein, P. G. Oviposition in the blood-sucking insect *Rhodnius prolixus* is modulated by host odors. Parasites & Vectors **8** (2015).
6. Riffell, J. A. *et al*. Flower discrimination by pollinators in a dynamic chemical environment. *Science* **344**, 1515–1518 (2014).
7. Lindh, J. M. *et al*. Discovery of an oviposition attractant for gravid malaria vectors of the Anopheles gambiae species complex. *Malaria Journal* **14** (2015).
8. Späthe, A. *et al*. Plant species- and status-specific odorant blends guide oviposition choice in the moth *Manduca sexta*. *Chemical Senses* **38**, 147–159 (2013).
9. Wondwosen, B. *et al*. Rice volatiles lure gravid malaria mosquitoes, *Anopheles arabiensis*. *Scientific Reports* **6** (2016).
10. Jaenike, J. On optimal oviposition behavior in phytophagous insects. *Theoretical Population Biology* **14**, 350–356 (1978).
11. Craig, T. P., Itami, J. K. & Price, P. W. A Strong relationship between oviposition preference and larval performance in a shoot-galling Sawfly. *Ecology* **70**, 1691–1699 (1989).
12. Valladares, G. & Lawton, J. H. Host-plant selection in the holly leaf-miner: Does mother know best? *The Journal of Animal Ecology* **60**, 227 (1991).
13. Ladner, D. T. & Altizer, S. Oviposition preference and larval performance of north american monarch butterflies on four *Asclepias* species. *Entomologia experimentalis et applicata* **116**, 9–20 (2005).
14. Clark, K. E., Hartley, S. E. & Johnson, S. N. Does mother know best? The preference-performance hypothesis and parent-offspring conflict in aboveground-belowground herbivore life cycles. *Ecological Entomology* **36**, 117–124 (2011).
15. Gómez Jiménez, M. I. *et al*. Oviposition, larval preference, and larval performance in two polyphagous species: does the larva know best? *Entomologia Experimentalis et Applicata* **153**, 24–33 (2014).
16. Kiflawi, M., Blaustein, L. & Mangel, M. Oviposition habitat selection by the mosquito *Culiseta longiareolata* in response to risk of predation and conspecific larval density. *Ecological Entomology* **28**, 168–173 (2003).
17. Reiskind, M. H., Greene, K. L. & Lounibos, L. P. Leaf species identity and combination affect performance and oviposition choice of two container mosquito species. *Ecological Entomology* **34**, 447–456 (2009).
18. Heard, S. B. Imperfect oviposition decisions by the pitcher plant mosquito (*Wyeomyia smithii*). *Evolutionary Ecology* **8**, 493–502 (1994).
19. Wong, J. *et al*. Linking oviposition site choice to offspring fitness in *Aedes aegypti*: Consequences for targeted larval control of Dengue vectors. *PLoS Neglected Tropical Diseases* **6**, e1632 (2012).
20. Dsouli-Aymes, N. *et al*. Global population structure of the stable fly (*Stomoxys calcitrans*) inferred by mitochondrial and nuclear sequence data. *Infection, Genetics and Evolution* **11**, 334–342 (2011).
21. Mihok, S., Munyoki, E. & Saleh, K. Phenology of Stomoxyinae in a Kenyan forest. *Medical and Veterinary Entomology* **10**, 305–316 (1996).
22. Broce, A. B. & Haas, M. S. Relation of cattle manure age to colonization by stable fly and house fly (Diptera: Muscidae). *Journal of the Kansas Entomological Society* 60–72 (1999).
23. Yeruhan, I. & Braverman, Y. Skin lesions in dogs, horses, and calves caused by the stable fly *Stomoxys calcitrans* (L.) (Diptera: Muscidae). *Revue de médecine vétérinaire des pays tropicaux*. **4**, 347–349 (1995).
24. Johnson, G., Panella, N., Hale, K. & Komer, N. Detection of west nile virus in stable flies (Diptera: Muscidae) parasitizing juvenile American white pelicans. *Journal of Medical Entomology* **47**, 1205–1211 (2010).
25. Hugh-Jones, M. & Blackburn, J. The ecology of *Bacillus anthracis*. *Molecular Aspects of Medicine* **30**, 356–367 (2009).
26. Sumba, A. L., Mihok, S. & Oyieke, F. A. Mechanical transmission of *Trypanosoma evansi* and *T. congolense* by *Stomoxys niger* and *S. taeniatus* in a laboratory mouse model. *Medical and Veterinary Entomology* **12**, 417–422 (1998).
27. Traversa, D. *et al*. Molecular diagnosis of equid summer sores. *Veterinary Parasitology* **150**, 116–21 (2007).
28. Taylor, D. B., Moon, R. D. & Mark, D. R. Economic impact of stable flies (Diptera: Muscidae) on Dairy and Beef Cattle Production. *Journal of Medical Entomology* **49**, 198–209 (2012).
29. Jeanbourquin, P. & Guerin, P. M. Chemostimuli implicated in selection of oviposition substrates by the stable fly *Stomoxys calcitrans*. *Medical and veterinary entomology* **21**, 209–216 (2007).

30. Hafez, M. & Gamal-Eddin, F. M. Ecological studies on *Stomoxys calcitrans* L. and *Sitiens* Rond, in Egypt, with suggestions on their control. *Bulletin de la Société Entomologique d' Egypte* XLIII 245–283 (1959)
31. Cançado, P. H., Ferreira, T., Piranda, E. M. & Soares, C. O. Sugarcane stems as larval habitat for the stable fly (*Stomoxys calcitrans*) in sugarcane plantations. *Pesquisa Veterinária Brasileira* **33**, 741–744 (2013).
32. Mansourian, S. *et al*. Fecal-derived phenol induces egg-laying aversion in *Drosophila*. *Current Biology* **26**, 2762–2769 (2016).
33. Marneweck, C., Jürgens, A. & Shrader, A. M. Temporal variation of white rhino dung odours. *Journal of Chemical Ecology* **43**, 955–965 (2017).
34. McCormick, A. C., Reinecke, A., Gershenzon, J. & Unsicker, S. B. Feeding experience affects the behavioral response of polyphagous gypsy moth caterpillars to herbivore-induced poplar volatiles. *Journal of Chemical Ecology* **42**, 382–393 (2016).
35. Perry, J. N., Wall, C. & Greenway, A. R. Latin square designs in field experiments involving insect sex attractants. *Ecological Entomology* **5**, 385–396 (1980).
36. Tangtrakulwanich, K. *et al*. Behavioural responses of stable flies to cattle manure slurry associated odourants: Stable fly attracted to manure slurry volatiles. *Medical and Veterinary Entomology* **29**, 82–87 (2015).
37. Frago, E. & Bauce, É. Life-history consequences of chronic nutritional stress in an outbreaking insect defoliator. *PloS one* **9**, e88039 (2014).
38. Rizvi, S. Z. M. & Raman, A. Effect of leaf chemistry of *Vitis vinifera* L. on the performance and development of *Epiphyas postvittana* (Lepidoptera: Tortricidae): Effect of grape cultivar on *Epiphyas postvittana* performance. *Australian Journal of Grape and Wine Research* **23**, 95–102 (2017).
39. Bhattacharya, A. & Kaliwal, B. B. The biochemical effects of potassium chloride on the silkworm, (Bombyx mori L.). *Insect Science* **12**, 95–100 (2005).
40. Schofield, R. M. S., Nesson, M. H. & Richardson, K. A. Tooth hardness increases with zinc-content in mandibles of young adult leaf-cutter ants. *Naturwissenschaften* **89**, 579-83 (2002)
41. Willott, E. & Tran, H. Q. Zinc and *Manduca sexta* hemocyte functions. *Journal of Insect Sciences.* **2**, 9 (2002).
42. Perkins, M. C., Woods, H. A., Harrison, J. F. & Elser, J. J. Dietary phosphorus affects the growth of larval *Manduca sexta*. *Archives of Insect Biochemistry and Physiology* **55**, 153–168 (2004).
43. Heisswolf, A., Obermaier, E. & Poethke, H. J. Selection of large host plants for oviposition by a monophagous leaf beetle: nutritional quality or enemy-free space? *Ecological Entomology* **30**, 299–306 (2005).
44. Chen, Y., Ruberson, J. R. & Olson, D. M. Nitrogen fertilization rate affects feeding, larval performance, and oviposition preference of the beet armyworm, *Spodoptera exigua*, on cotton. *Entomologia Experimentalis et Applicata* **126**, 244–255 (2008).
45. Staley, J. T., Stewart-Jones, A., Poppy, G. M., Leather, S. R. & Wright, D. J. Fertilizer affects the behaviour and performance of *Plutella xylostella* on brassicas. *Agricultural and Forest Entomology* **11**, 275–282 (2009).
46. Pagadala Damodaram, K. J. *et al*. Oviposition site-selection by *Bactrocera dorsalis* is mediated through an innate recognition template tuned to γ-octalactone. *PLoS ONE* **9**, e85764 (2014).
47. Kamala Jayanthi, P. D. *et al*. Specific volatile compounds from mango elicit oviposition in gravid *Bactrocera dorsalis* females. *Journal of Chemical Ecology* **40**, 259–266 (2014).
48. Logan, J. G. & Birkett, M. A. Semiochemicals for biting fly control: their identification and exploitation. *Pest Management Science* **63**, 647–657 (2007).
49. Norin, T. Semiochemicals for insect pest management. *Pure and Applied Chemistry* **79** (2007).
50. Reisenman, C. E., Lei, H. & Guerenstein, P. G. Neuroethology of olfactory-guided behavior and its potential application in the control of harmful insects. *Frontiers in Physiology* **7** (2016).
51. Mihok, S., Kang'ethe, E. K. & Kamau, G. K. Trials of Traps and attractants for *Stomoxys* spp. (Diptera: Muscidae). *Journal of Medical Entomology* **32**, 283–289 (1995).
52. Tunnakundacha, S., Desquesnes, M. & Masmeatathip, R. Comparison of Vavoua, Malaise and Nzi traps with and without attractants for trapping of Stomoxys spp. (Diptera: Muscidae) and tabanids (Diptera: Tabanidae) on cattle farms. *Agriculture and Natural Resources* https://doi.org/10.1016/j.anres.2017.07.002 (2017).
53. Sommer, C. *et al*. Ivermectin excreted in cattle dung after subcutaneous injection or pour-on treatment: concentrations and impact on dung fauna. *Bulletin of Entomological Research* **82**, 257–264 (1992).
54. Bremner, J. M. Nitrogen total. In Sparks D. L., editors. Methods of soil analysis, Part 3 chemical methods. Soil Science Society of America, American Society of Agronomy, Madison, WI, 1085–1121 (1996).
55. Matthiessen, M. K., Larney, F. J., Brent Selinger, L. & Olson, A. F. Influence of Loss-on- Ignition Temperature and Heating Time on Ash Content of Compost and Manure. *Communications in Soil Science and Plant Analysis* **36**, 2561–2573 (2005).
56. Boss, C. B. & Fredeen, K. J. Concepts, Instrumentation and Techniques in Inductively Coupled Plasma Optical Emission Spectrometry. 120.
57. Tholl, D. *et al*. Practical approaches to plant volatile analysis. *The Plant Journal* **45**, 540–560 (2006).
58. Breiman, L. Random forests. *Machine learning* **45**, 5–32 (2001).
59. Ranganathan, Y. & Borges, R. M. Reducing the babel in plant volatile communication: using the forest to see the trees: Random Forest-based volatile selection. *Plant Biology* **12**, 735–742 (2010).
60. Liaw, A. & Wiener, M. Classification and regression by randomForest. *R news* **2**, 18–22 (2002).
61. Marneweck, C., Jürgens, A. & Shrader, A. M. Dung odours signal sex, age, territorial and oestrous state in white rhinos. *Proceedings of the Royal Society B: Biological Sciences* **284**, 20162376 (2017).
62. Bengtsson, J. M. *et al*. Field attractants for *Pachnoda interrupta* selected by means of GC-EAD and single sensillum screening. *Journal of Chemical Ecology* **35**, 1063–1076 (2009).
63. Zumpt, F. The Stomoxyine biting flies of the world: Diptera, Muscidae; taxonomy, biology, economic importance and control measures. Gustav Fischer Verlag, Stuttgart, Germany (1973).
64. R Core Team. R: A language and environment for statistical computing R Foundat on for Statistical Computing, Vienna, Austria, https://www.R-project.org/ (2018)
65. McDonald, J. H. *Handbook of biological statistics*. **2**, (Sparky House Publishing Baltimore, MD 2009).
66. Warton, D. I. & Hui, F. K. The arcsine is asinine: the analysis of proportions in ecology. *Ecology* **92**, 3–10 (2011).
67. Lenth R. lsmeans: Least-squares means. R Package Version 2.20-23 (2016)
68. de Mendiburu, F. agricolae tutorial (Version1.2-8) (2017).
69. Kassambara, A. Practical Guide to Principal Component Methods in R. 170
70. Hout, M. C., Papesh, M. H. & Goldinger, S. D. Multidimensional scaling. *Wiley Interdisciplinary Reviews: Cognitive Science* **4**, 93–103 (2013).
71. Venables, W. N. & Ripley, B. D. Modern applied statistics with S (4th ed). New York: Springer (2002).
72. Bretz, F., Hothorn, T. & Westfall, P. H. *Multiple comparisons using R*. (CRC Press 2011).

Acknowledgements

This work was supported by the IBCARP camel, grant no. DCI-FOOD/2014/ 346–739 - mainly by European Union. We also gratefully acknowledge the financial support for this research by the following organizations and agencies: Swedish International Development Cooperation Agency (Sida); UK Department for International Development (DFID); the Swiss Agency for Development and Cooperation (SDC); and the Kenyan Government. The views expressed herein do not necessarily reflect the official opinion of the donors. The Deutscher Akademischer Austauschdienst (DAAD) provided Steve B. S. Baleba with a doctoral scholarship through the *icipe* ARPPIS-DAAD scholarship programme. We are grateful to the Max Planck Institute for Chemical Ecology for providing us with some standard chemicals. We wish to thank Peter Ahuya, Abel Orone and John Ngiela for technical support and Caroline Muya for administrative assistance. We thank Mpala Research Centre and Ranch for allowing us to do our field experiment. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Author Contributions

Steve B.S. Baleba: Study conceptualisation, experimental design, data collection, data analysis, manuscript preparation; Baldwyn Torto: supervision and manuscript preparation; Daniel Masiga: supervision and manuscript preparation; Christopher W. Weldon: supervision, experimental design and manuscript preparation; Merid N. Getahun: study conceptualisation, experimental design, supervision, manuscript preparation.

Additional Information

Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Parasites & Vectors

RESEARCH Open Access

Effect of larval density and substrate quality on the wing geometry of *Stomoxys calcitrans* L. (Diptera: Muscidae)

Steve B. S. Baleba[1,2*], Daniel Masiga[1], Baldwyn Torto[1,2], Christopher W. Weldon[2] and Merid N. Getahun[1]

Abstract

Background: In insects, oviposition decisions may lead to egg deposition in substrates with different larval density and nutritional levels. Individuals developing in such substrates may present plasticity in their phenotype. Here, we investigated the effect of two factors related to oviposition decisions, namely larval density and substrate quality, on the wing size and wing shape of the stable fly, *Stomoxys calcitrans* L. (Diptera: Muscidae).

Methods: We reared *S. calcitrans* larvae at different densities (5, 15 and 25) and on different substrates (camel, cow, donkey and sheep dung). For each fly that emerged, we recorded body weight, and detached, slide-mounted and photographed the right wing. Next, we collected 15 landmarks on each photographed wing, and applied geometric morphometric analysis to assess variation in wing size and wing shape of *S. calcitrans* across the different larval densities and substrate types.

Results: We observed that wing size and wing shape of *S. calcitrans* were affected by larval density and the nature of the developmental substrate. Flies reared in a group of 5 had larger wing centroid size, wing length, wing width, wing area and wing loading compared with those reared in a group of 25. Also, flies developed in donkey and sheep dung had larger wing centroid size, wing length, wing width, wing area and wing loading in comparison with those grown in camel and cow dung. Canonical variate analysis followed by discriminant analysis revealed significant wing shape variation in *S. calcitrans* across the different densities and substrates. Wing size had a significant but weak positive effect on wing shape.

Conclusions: This study demonstrates the high sensitivity of *S. calcitrans* wings to variation in larval density and developmental substrate, and that use of landmark-based geometric morphometric analysis could improve our understanding of how flies of veterinary importance respond to environmental variability.

Keywords: *Stomoxys calcitrans*, Wing morphology, Larval density, Developmental substrate, Geometric morphometrics, Phenotypic plasticity

Background

In holometabolous insects, individual fitness mostly relies on oviposition decisions by gravid females. When, where and how mothers deposit their eggs can affect the performance and phenotype of their progeny [1]. Therefore, it is important for females to oviposit on a substrate that provides the best conditions for the next generation. However, a female may fail to make the seemingly optimal choice to oviposit on an appropriate substrate that could enhance offspring fitness. For instance, Heard [2] found that in the pitcher plant mosquito, *Wyeomyia smithii* Coquillett, although larval fitness is better in pitchers with fewer conspecific and more midge larvae, gravid females did not deposit more eggs in such pitchers. Instead, they laid more eggs in pitchers containing either midges or conspecific larvae. Wong et al. [3] found that larval survival and development of *Aedes aegypti* L. was poor in containers where gravid females laid more

*Correspondence: bbaleba@icipe.org; sbernardsteve@gmail.com
[1] International Centre of Insect Physiology and Ecology (icipe), P.O. Box 30772-00100, Nairobi, Kenya
Full list of author information is available at the end of the article

eggs. This imperfection in oviposition decisions generally leads to phenotypic variation [4, 5], in which individuals react to the inputs of their breeding substrate with a change in their form, state, movement, or rate of activity [6]. These inputs include environmental factors such as the dietary value of the substrate and the number of individuals sharing the same substrate (density) [7]. In evolutionary ecology, understanding how these factors influence organism phenotype is a fundamental concern because such flexibility can affect fitness, generate novelty, facilitate evolution, and structure ecological communities [8].

Insect wings are good indicators of population responses to changes that occur in their environment [9]. Lin et al. [10] demonstrated that variation in food nutrient content and density are key ecological factors related to the expression of condition-dependent, adaptive phenotypes such as wing polyphenisms. They found that in the brown planthopper, *Nilaparvata lugens* Stål, a serious rice pest, an increase of long-winged *N. lugens* in a population is related to higher glucose levels in host rice plants. Conversely, the appearance of the short-winged form of *N. lugens* is linked to a reduction in host glucose level. Dipteran species are particularly well suited for studying phenotypic changes induced by the environment because their wings are highly plastic and wing landmarks are homologous across various species [11]. Variation in food quality and population density are key factors associated with fly wing polyphenisms. In *Drosophila buzzatii* Patterson & Wheeler and *Drosophila koepferae* Soto et al. [12] detected significant differences in wing size and shape between flies that were reared on different cactus hosts. In *Ae. aegypti*, males and females have longer wings when developed in conditions of low larval density [13].

Changes in wing morphology are known to affect insect flight aerodynamics. Long and slender wings are optimal for long-duration flight, while short and broad wings are optimal for slow and agile flight [14]. Also, broad wing bases allow a wider range of speed and a narrow wing tip allows less costly, extensive flight [15]. As a consequence, wing morphology is closely related to several insect behavioural activities including food searching, location of breeding sites and sexual partners, and avoidance of natural enemies. In some mosquito species such as *Anopheles gambiae* Giles where wing size is positively correlated with body size, an increase in wing size augments the frequency of blood meals [16]. This then leads to an increase in the likelihood of pathogen dissemination [16]. In *Aedes albopictus* Skuse, there is a positive correlation between wing length, larval diet quality, and the number of eggs laid [17]. It has been demonstrated that males of the olive fruit fly, *Bactrocera oleae* Rossi, with large wings (characterised by a high vibration frequency) achieve higher mating success than males with smaller wings [18].

Fly wing vein networks are excellent models for statistical analysis of size and shape variation [19]. In recent years, landmark-based geometric morphometric analysis has been increasingly used to analyse insect wings to address intraspecific variation [20, 21], interspecific variation [22, 23], sexual dimorphism [24, 25], parasite detection [26, 27], laboratory strain separation [28] and phenotypic plasticity [12, 29, 30]. Geometric morphometric techniques are potent tools to assess the correlation between the size and shape of organisms and environmental variables. The approach uses coordinates of identified morphological "landmarks" to study the form of biological structures in two or three dimensions. It involves several statistical techniques that preserve shape information and detect even subtle morphological variations [31]. Moreover, geometric morphometric techniques are cheap, simple and fast [32]. Using geometric morphometric analysis, this study examined the changes that occur in the wings of the stable fly, *Stomoxys calictrans* L. (Diptera: Muscidae) reared on different substrates and over a range of larval densities.

Stomoxys calcitrans is a cosmopolitan haematophagous fly that mechanically transmits 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, which include cattle, camels, horses, dogs, and humans [33–35]. During outbreaks, *S. calcitrans* can reduce weight gain in cattle by up to 19%, and lead to a 40–60% reduction in milk yields [36, 37]. In the USA, Taylor et al. [38] estimated economic losses attributable to *S. calcitrans* infestation at around $2.211 billion per year. Gravid female *S. calcitrans* oviposit on vertebrate herbivore dung including that of camel, cow, donkey and sheep, with the latter two the most preferred [39]. It has already been demonstrated that the fitness of *S. calcitrans* immature stages (hatchability, developmental time, emergence time, larval and pupal weight) varies across these substrates due to differences in their physicochemical composition [39]. However, the way in which preferred and non-preferred substrates affect *S. calcitrans* wing size and wing shape remains unclear. Furthermore, not only the substrate nutrient quality but also larval density should be assessed. We hypothesised that larval density and vertebrate herbivore dung type on which *S. calcitrans* develop would affect wing size and shape.

Methods

Biological material

Stomoxys calcitrans flies were obtained from a single culture that had been established for approximately 8 months at the Duduville campus of the International Centre for Insect Physiology and Ecology (icipe) in Nairobi (1°13′12″S, 36°52′48″ E; *c.*1600 m above sea level). By sourcing experimental flies from a laboratory culture, we minimised potential variation between populations. Adults reared from rabbit faeces were kept in cages (75 × 60 × 45 cm) under conditions of 25 ± 5 °C and 65 ± 5% relative humidity with a photoperiod of 12L:12D. Flies were fed twice per day (8:00 and 16:00 h) on defibrinated bovine blood poured on moistened cotton.

Density experiment

Gravid female *S. calcitrans* from the established colony were allowed to oviposit on donkey dung placed in plastic containers (21.5 × 14.5 × 7.4 cm). Baleba et al. [39] demonstrated that this dung is best for *S. calcitrans* development. To assess the effect of density of *S. calcitrans* on wing size and shape, we reared *S. calcitrans* larvae at varying densities by gently transferring (using soft forceps) 5, 15 and 25 first-instar larvae to plastic cups (200 ml) filled with 25 g of donkey dung. We replicated this process several times to obtain 30 emerged females and 30 emerged males. After emergence, each individual was weighed, killed in 70% ethanol, and its right wing was gently removed from the thorax using a fine clamp. The removed wings were slide-mounted (dorsally placed between two microscope slides) to avoid deformation and to enhance accuracy during photography and landmark collections [40]. We photographed the wings at 16× magnification with a Leica DFC320 digital camera coupled to a Leica S6 microscope.

Substrate quality experiment

To test the effect of substrate on *S. calcitrans* wing size and shape, 10 first-instar larvae were transferred to and permitted to develop on 25 g of camel, cow, donkey or sheep dung. It has been shown that these substrates differ in their physico-chemical composition and affect development and adult body weight [39]. We replicated the process several times to obtain 30 emerged females and 30 emerged males. After emergence, each individual of *S. calcitrans* was weighed, killed in 70% ethanol, the right wing was removed and slide-mounted, and wings were photographed as described above.

Wing geometric morphometric analysis

To collect wing landmark coordinates, we opened the digital photographs in ImageJ software [41] and generated Cartesian coordinates for 15 wing landmarks (Fig. 1). To quantify measurement error relative to the landmark digitalisation, we collected landmarks for the wings of all 30 individuals reared from donkey dung three times. After executing the generalised Procrustes analysis to extract shape information from the data and eliminate differences in orientation, position and isometric size, we ran analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA) tests to determine if wing size and the wing shape of *S. calcitrans* varied across the three landmark collections [42] (Table 1).

Four parameters were derived to describe wing size of *S. calcitrans*: (i) centroid size; (ii) wing length (distance between the 6th and 12th landmark); (iii) wing width (distance between the 3rd and 8th landmark) (Fig. 1); and (iv) wing area. The centroid size, also called the "configuration barycentre" is a global size (or multidimensional measurement) calculated as the square root of the sum of squared Euclidean distances between each landmark and the wing centroid. We computed centroid

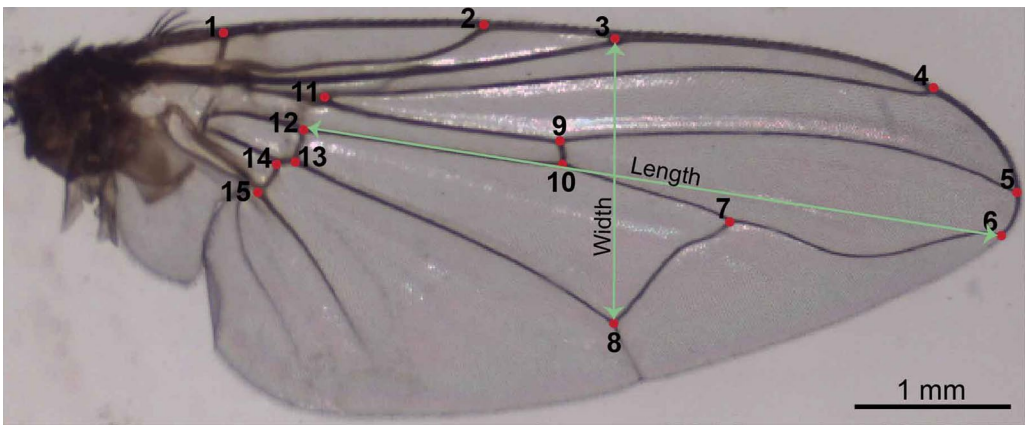

Fig. 1 Dorsal view of the right wing of *S. calcitrans*. Numbers indicate the location of 15 selected landmarks (described in Table 1)

Table 1 Description of the 15 anatomical landmarks used to characterise *S. calcitrans* wing geometry. Numbers relate to landmarks shown in Fig. 1

Anatomic position of landmark	Description
1	Costal vein intersection with humeral vein
2	Costal vein intersection with subcosta vein
3	Costal vein intersection with radial vein 1
4	Costal vein intersection with radial vein 2+3
5	Costal vein intersection with radial vein 4+5
6	Costal vein intersection Medial vein
7	Basal median cubital vein intersection medial vein
8	Basal median cubital vein and anterior cubital 2
9	Radial vein 4+5 vein intersection with radial medial
10	Medial vein intersection with radial medial vein
11	Radial 2+3 vein intersection with radial 4+5 vein
12	Medial vein intersection with distal median cubital
13	Anterior cubital vein 1 intersection with distal median cubital
14	Anterior cubital vein 1 intersection with anterior cubital vein 2
15	Anal vein 1 intersection with anterior cubital vein 2

size using PAST software V.3.09 [43]. Also, based on the adult weight parameter, we calculated wing loading (in kg/m^2) using the formula: wl = mass/wing area [44]. To assess the effect of larval density (5, 15 and 25) and substrate type (camel, cow, donkey and sheep dung) on the parameters described above, we ran analyses of variance (ANOVA) followed by *post-hoc* Student-Newman-Keuls (SNK) tests after checking the wing size parameters for normality using the Shapiro-Wilk test (P > 0.05). To identify correlations between centroid size, wing length, wing width, wing area, adult weight and wing loading, we performed separate principal components analysis (PCA) for larval density and substrate type. We used R version 3.5.1 software [45] to compute all statistical analyses.

To assess wing shape variation across the different densities and substrates, we imported the raw landmark Cartesian coordinates into MorphoJ software [46]. This software was first used to perform a generalised Procrustes analysis to extract shape information from the data and eliminate differences in orientation, position and isometric size. Afterwards, we ran separate multivariate analyses of variance (MANOVA) to compare wing shapes across the different larval densities (5, 15 and 25) and substrates (camel, cow, donkey and sheep dung). Using PAST software, we performed thin plate spline analysis to visualise wing shape deformations. We used canonical variate analysis combined with discriminant analysis to analyse the relative similarities and dissimilarities of the different wing groups. To determine the significance of pairwise differences in mean shapes, we performed permutation tests (10,000 rounds) with Mahalanobis distances and Procrustes distances. To assess the effect of wing size on wing shape (allometry), we fit a linear regression between the Procrustes coordinates and the centroid size, using a permutation test with 10,000 randomisations. For all of these analyses, we excluded the effect of sex because preliminary analyses indicated that wing shape of females and males did not differ.

Results

Measurement error test

The landmarks measured repeatedly on the same individual wing (3 times) were not significantly different for both size (ANOVA, $F_{(2,84)} = 0.03$; $P = 0.97$) and shape (MANOVA, $F_{(48,2016)} = 0.6$; Pillai's trace = 0.16; $P = 0.99$). Therefore, we ruled out error due to landmark digitalisation, and we considered that any differences found in the morphology of *S. calcitrans* wings resulted from the two factors manipulated in our study (larval density and substrate type).

Effect of larval density on the wing geometry of *S. calcitrans*

Wing size parameters

Larval density significantly affected wing centroid size ($F_{(2,84)} = 104.1$, $P < 0.0001$), wing length ($F_{(2,84)} = 97.91$, $P < 0.0001$), wing width ($F_{(2,84)} = 85.63$, $P < 0.0001$), wing area ($F_{(2,84)} = 22.67$, $P < 0.0001$), adult weight ($F_{(2,84)} = 4.51$, $P = 0.014$) and wing loading ($F_{(2,84)} = 14.35$, $P < 0.0001$) of *S. calcitrans*. We obtained the largest wing centroid size (Fig. 2a.i), wing length (Fig. 2a.ii), wing width (Fig. 2a.iii), wing area (Fig. 2a.iv), adult weight (Fig. 2a.v) and wing loading (Fig. 2a.vi) in flies reared from a group of five larvae. The biplot from the principal component analysis separated flies reared in a group of five from those reared in a group of 25; flies reared in a group of 15 occupied an intermediate position (Fig. 2a). The two first dimensions accounted for 85.6% of the total wing size variation. Dimension 1 explained 60.6% of the total variation, with wing length as the major contributor. Dimension 2 accounted for 25% of the total variation, with wing loading as the major contributor. Except for an absence of correlation between adult weight and centroid size ($r = 0.18$, $P = 0.094$), and adult weight and wing area ($r = 0.02$, $P = 0.83$), all other parameters were significantly correlated either negatively or positively (Table 2). For instance, wing loading was positively correlated with adult weight ($r = 0.50$, $P < 0.0001$) and negatively correlated with wing width ($r = -0.43$, $P < 0.0001$), wing length ($r = -0.46$, $P < 0.0001$), centroid size ($r = -0.50$, $P < 0.0001$), and wing area ($r = -0.60$, $P < 0.0001$).

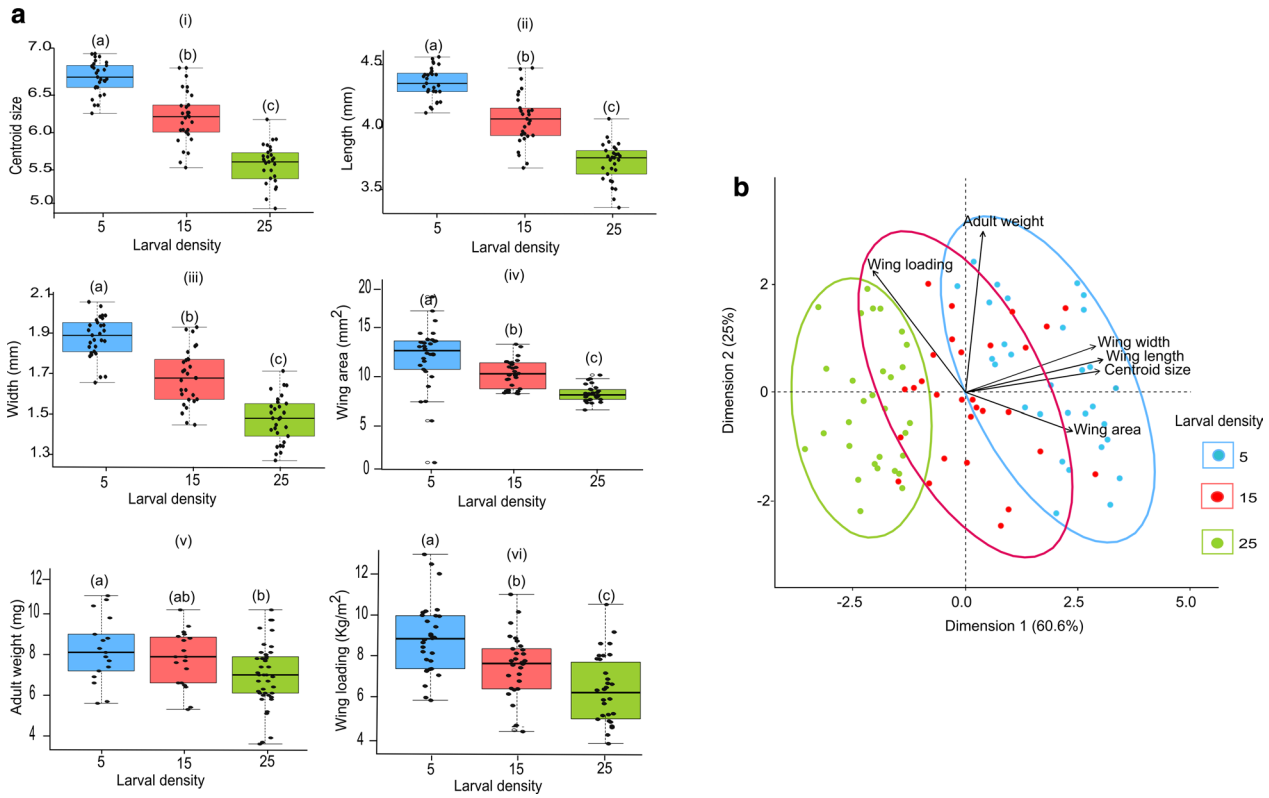

Fig. 2 Wing size and adult weight of *S. calcitrans* is significantly affected by larval density. **a** Boxplots depicting variation in wing centroid size (i), wing length (ii), wing width (iii), wing area (iv), adult weight (v), and wing loading (vi) across the different larval densities. Boxplot whiskers indicate ± 1.5 interquartile range limits. Boxplots with different letters show significant differences as grouped by ANOVA tests followed by SNK *post-hoc* tests ($P < 0.05$, $n = 30$). **b** Principal components biplot showing the similarities (or dissimilarities) existing among flies reared at different densities (5, 15 and 25)

Table 2 Correlation matrix between body weight and wing size parameters of *S. calcitrans* reared at different densities. Values above the diagonal represent *P*-values from Pearson correlation tests; values below the diagonal represent correlation coefficient. *P*-value in bold is not significant

	Wing area	Adult weight	Wing width	Wing length	Wing loading	Centroid size
Wing area	–	0.83	< 0.001	< 0.001	< 0.001	< 0.001
Adult weight	0.02	–	0.016	0.024	< 0.001	**0.094**
Wing width	0.54	0.25	–	< 0.001	< 0.001	< 0.001
Wing length	0.57	0.24	0.97	–	< 0.001	< 0.001
Wing loading	− 0.6	0.49	− 0.43	− 0.46	–	< 0.001
Centroid size	0.55	0.18	0.89	0.93	− 0.49	–

Wing shape parameters

The wing shape of *S. calcitrans* significantly differed between the larval densities (MANOVA, $F_{(52,1872)} = 2.26$; Pillai's trace = 0.99; $P = 0.0059$). The thin plate spline (Fig. 3a) showed the variation in expansions and contractions in wing vein intersections (landmarks) of flies emerged from densities of 5, 15 and 25. For instance, in flies from densities of 5 and 15, the landmarks 4, 5 and 6 underwent expansion movement, while in the flies from a density of 25, the same landmarks contracted. Additionally, in flies from densities of 5 and 25, expansions in the landmarks 12, 13 and 14 were more pronounced (red coloured) compared to those of flies from a density of 15 (yellow coloured). The landmarks 7, 8, 9 and 10 expanded in wings emerged from a density of 15, while in fly wings from densities of 5 and 25, these landmarks contracted.

Canonical variate analysis discriminated flies emerged from each density based on wing shape (Fig. 3b). The two first dimensions accounted for 100% of the total shape variation (CV1 = 82.56% and CV2 = 17.44%), and clustered wing shapes in three distinct groups based on the three larval densities. Pairwise comparisons using discriminant analysis with Mahalanobis distances revealed a highly significant difference in *S. calcitrans* wing shapes (Fig. 3c; Table 3; permutation test, 10000 replicates, $P < 0.0001$). When Procrustes distances were used, we found that wing shape of flies reared from densities of 15 and 25 were similar ($P = 0.16$). Regression of Procrustes coordinates on centroid size between densities was significant (permutation test with 10000 rounds, $P = 0.008$), with allometry explaining 2.97% of the total shape variation.

Effect of developmental substrate on the wing geometry of *S. calcitrans*

Wing size parameters

Wing centroid size (Fig. 4a.i; $F_{(3,122)} = 39.24$, $P < 0.0001$), wing length (Fig. 4a.ii; $F_{(3,122)} = 34.9$, $P < 0.0001$), wing width (Fig. 4a.iii; $F_{(3,122)} = 46.98$, $P < 0.0001$), wing area (Fig. 4a.iv; $F_{(3,122)} = 31.02$, $P < 0.0001$), adult weight (Fig. 4a.v; $F_{(3,122)} = 140.4$, $P < 0.0001$) and the wing loading (Fig. 4a.vi; $F_{(3,122)} = 67.79$, $P < 0.0001$) of *S. calcitrans* individuals reared from various animal dung differed significantly. All these parameters were highest in flies reared on donkey and sheep dung. The principal components analysis differentiated flies emerged from the different herbivore dung (Fig. 4b). The first dimension accounted for 55.5% of the variation in wing shape and were highly correlated with adult weight. The second

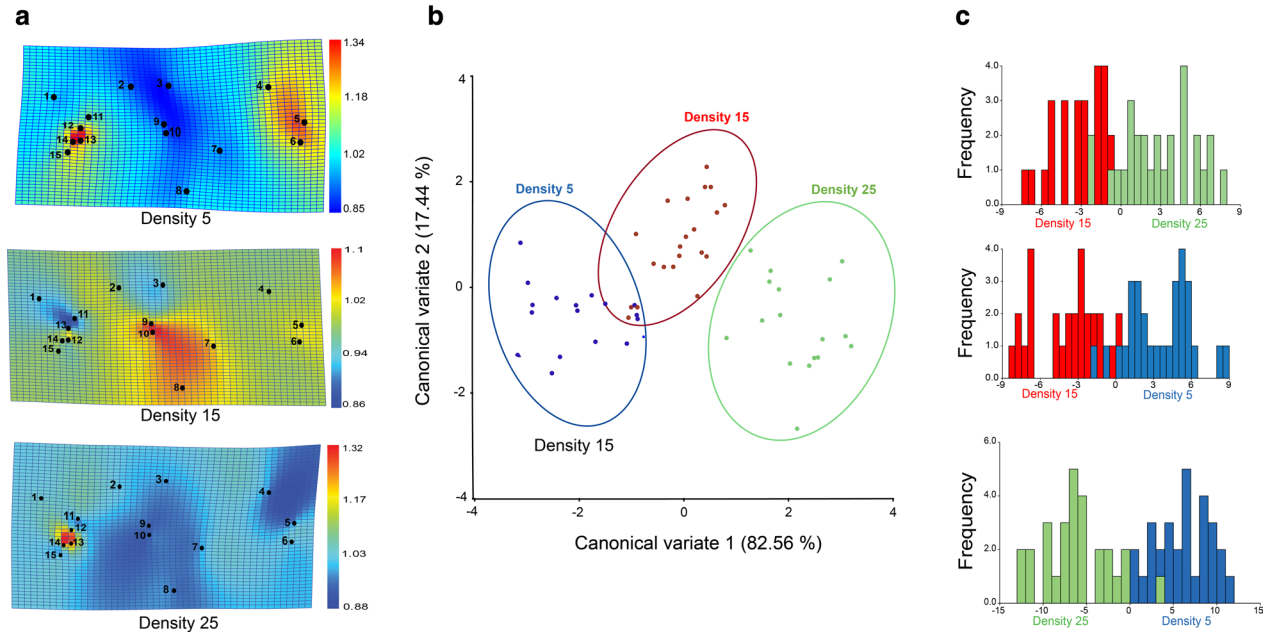

Fig. 3 Wing shape of *S. calcitrans* is significantly affected by larval density. **a** Thin plate spline deformation grids modelling the difference of *S. calcitrans* wing shape across the different larval densities. The number displayed on each grid represents the landmark positions. Yellow to orange-red colours indicate landmark expansions, light to dark-blue indicates landmark contraction. **b** Scatter plot showing the difference in wing shapes of *S. calcitrans* individuals reared at different larval densities along the first two canonical variate axes [CV1 (82.56%) and CV2 (17.44%)] with 90% confidence ellipses. **c** Discrimination histograms comparing the *S. calcitrans* wing shape between pairs of larval densities

Table 3 Difference in the shape of right wings from *S. calcitrans* reared at a density 5, 15 and 25. *P*-values (above the diagonal); distances between populations (below the diagonal). $P < 0.05$ denotes a significant difference

	Mahalanobis distances			Procrustes distances		
	Density 5	Density 15	Density 25	Density 5	Density 15	Density 25
Density 5	–	< 0.001	< 0.001	–	0.0471	< 0.001
Density 15	2.01	–	< 0.001	0.0086	–	0.16
Density 25	3.38	2.23	–	0.0134	0.0083	–

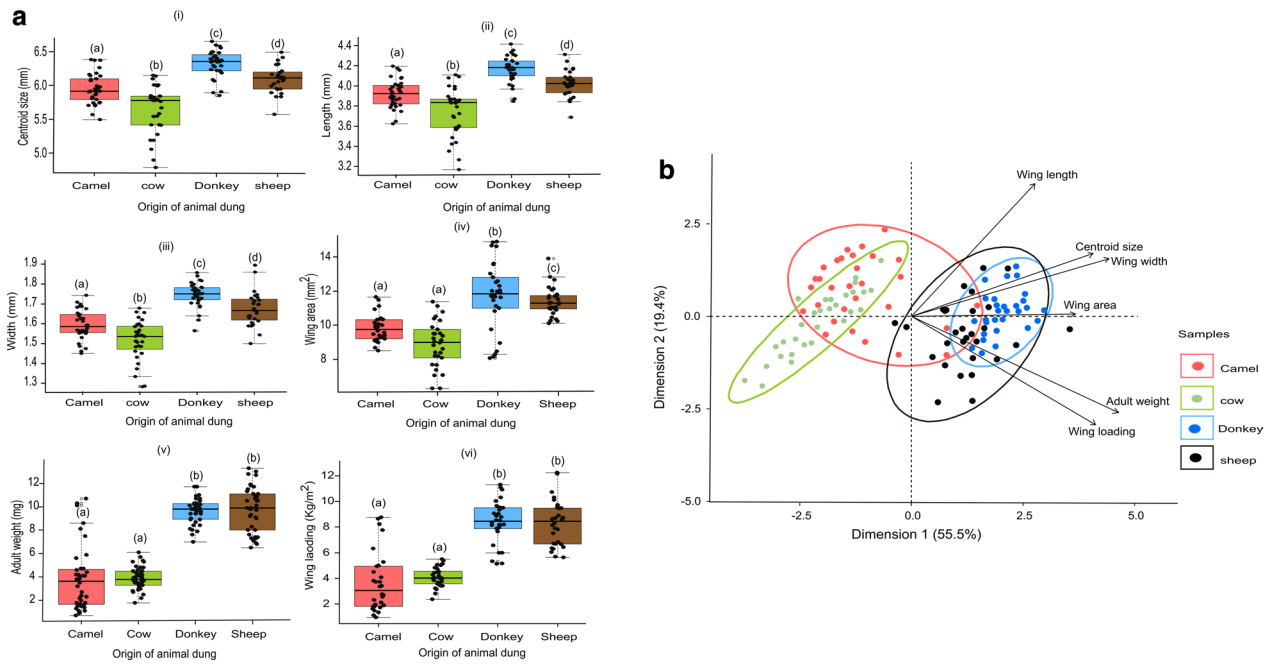

Fig. 4 Wing size and adult weight of *S. calcitrans* significantly varies across different developmental substrates. **a** Boxplots depicting variation in wing centroid size (i), wing length (ii), wing width (iii), wing area (iv), adult weight (v), and wing loading (vi) across the different dung substrates. The limit of each boxplot whiskers represents the minimum and maximum of all the data. Boxplots with different letters depict significant differences as grouped by ANOVA tests followed by SNK *post-hoc* tests ($P < 0.05$, $n = 30$). **b** Principal components biplot showing the similarities (or dissimilarities) existing among flies reared from different dung types

Table 4 Correlation matrix between body weight and wing size parameters of *S. calcitrans* reared from different developmental substrates. Values above the diagonal represent *P*-values from Pearson correlation tests; values below the diagonal represent correlation coefficient. *P*-value in bold is not significant

	Centroid size	Wing length	Wing width	Wing area	Wing loading	Wing mass
Centroid size	1	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Wing length	0.43	1	< 0.001	0.01	**0.08**	0.035
Wing width	0.66	0.52	1	< 0.001	< 0.001	< 0.001
Wing area	0.46	0.23	0.49	1	< 0.001	< 0.001
Wing loading	0.39	0.16	0.47	0.3	1	< 0.001
Wing mass	0.46	0.19	0.54	0.57	0.95	1

dimension explained 19.4% of the total variation and was highly associated with wing length. Other than a lack of correlation between wing loading and wing length ($r = 0.16$, $P = 0.08$), all wing size parameters were positively correlated (Table 4).

Wing shape parameter

Developmental substrate significantly affected the wing shape of *S. calcitrans* (MANOVA, $F_{(78,3172)} = 4.07$, Pillai's trace = 1.17, $P < 0.0001$). This is clearly illustrated in the thin plate spline deformation grid (Fig. 5a). For instance, in flies emerged from camel dung (Fig. 5a.i), landmarks 12, 13, 14 and 15 underwent expansion movement, whereas in flies from cow (Fig. 5a.ii), donkey (Fig. 5a.iii) and sheep (Fig. 5a.iv) dung, the same landmarks contracted. Canonical variate analysis (Fig. 5b) and discriminant analysis (Fig. 5c) separated *S. calcitrans* wing shapes according to the dung in which flies developed. The first two dimensions of the canonical variate analysis explained 85.06% of the total *S. calcitrans* wing shape variation (Fig. 5b; CV1 = 66.06% and CV2 = 16%). All pairwise permutation tests performed with Mahalanobis

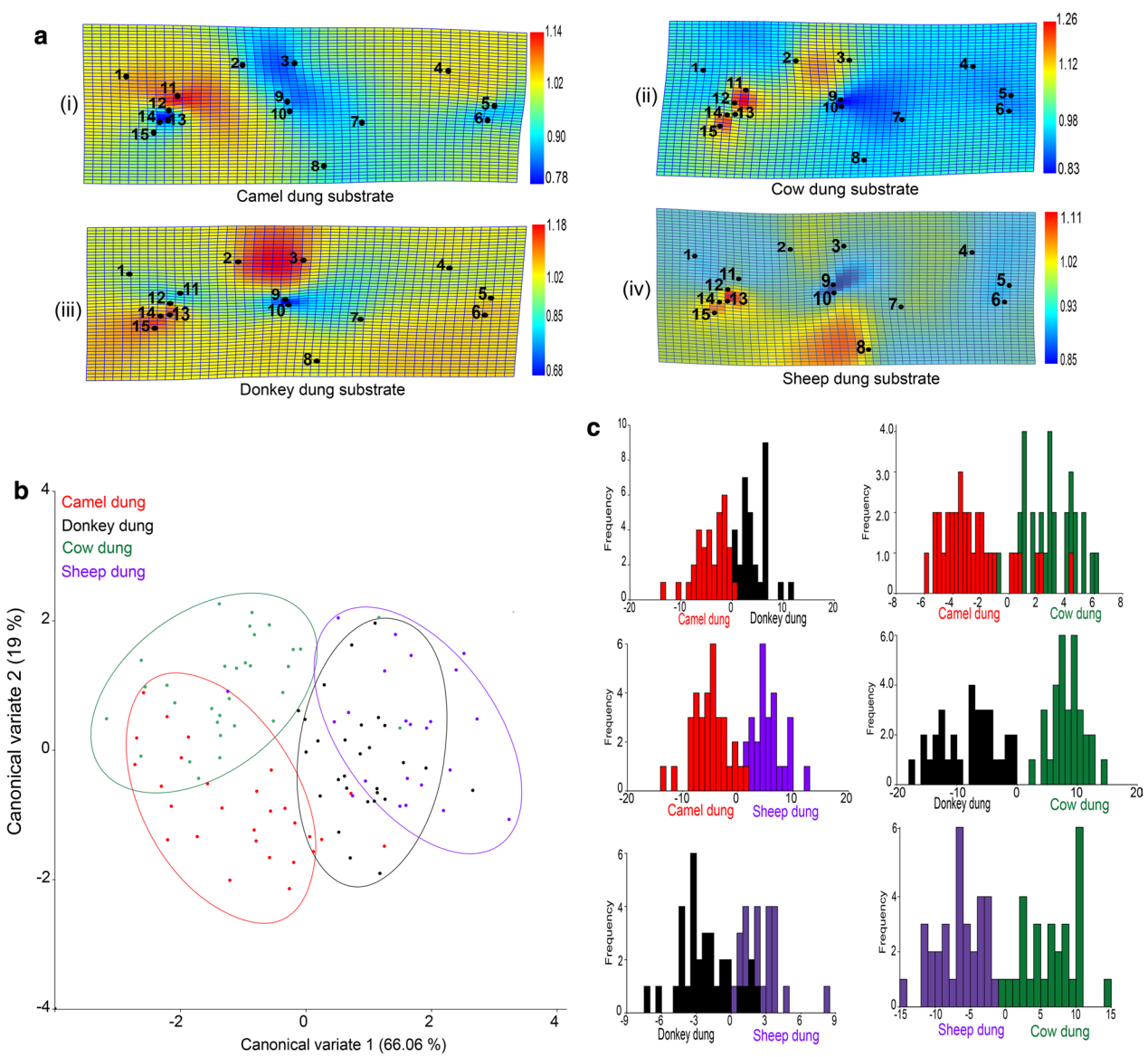

Fig. 5 Wing shape in *S. calcitrans* is significantly affected by developmental substrate. **a** Thin plate spline (TPS) deformation grids modelling the difference of *S. calcitrans* wing shape across the different larval densities. The number displayed on each grid represents the landmark positions. Yellow to orange-red colours indicate landmark expansions, light to dark-blue indicates landmark contraction. **b** Scatter plot showing the difference in wing shapes of *S. calcitrans* individuals reared from different developmental substrates along the first two canonical variate axes [CV1 (66.06%) and CV2 (19)] with 90% confidence ellipses. **c** Discrimination histograms comparing *S. calcitrans* wing shape between developmental substrate pairs

distances revealed that the shape of *S. calcitrans* wings diverged significantly when reared from the different animal dung (Table 5; 10,000 rounds, $P<0.0001$). With Procrustes distance estimators, we obtained a non-significant difference in wing shapes only in flies emerged from camel and cow dung ($P=0.2$). In the allometry test, the centroid size had a significant effect on wing shape (10,000 rounds of permutation tests, $P=0.0015$), with a variance prediction of 2.45%.

Discussion

Our study showed that the size and shape of *S. calcitrans* wings exhibit a plastic response to larval density and the quality of the larval substrate. The study also demonstrated the power of the wing landmark-based geometric approach for studying phenotypic plasticity.

We showed that *S. calcitrans* wing size parameters (centroid size, length, width, area, and loading) are affected by larval density (5, 15 and 25) and substrate

Table 5 Difference in the shape of right wings from *S. calcitrans* reared from various animal dung. *P*-values (above the diagonal); distances between populations (below the diagonal). $P < 0.05$ denotes significant difference

	Mahalanobis distances				Procrustes distances			
	Camel dung	Cow dung	Donkey dung	Sheep dung	Camel dung	Cow dung	Donkey dung	Sheep dung
Camel dung	–	< 0.0001	< 0.0001	< 0.0001	–	0.20	0.0002	< 0.0001
Cow dung	1.7975	–	< 0.0001	< 0.0001	0.0067	–	< 0.0001	< 0.0001
Donkey dung	2.2631	2.3678	–	< 0.0001	0.0109	0.0125	–	0.0476
Sheep dung	3.0651	3.1064	1.9544	–	0.0155	0.0153	0.0081	–

type (camel, cow, donkey and sheep dung). This indicates the effect of larval developmental conditions on adult wing size. Our study is consistent with previous work on *Ae. aegypti* that revealed the influence of larval density and substrate quality on wing size [47, 48]. The wing size variation obtained in our study may be due to variability in nutrients. Baleba et al. [39] previously determined that donkey and sheep dung, from which emerging flies had the largest wing size, had higher concentrations of specific micronutrients (nitrogen, phosphorous, potassium and zinc) in comparison with camel and cow dung. Furthermore, competition for limited nutrients by *S. calcitrans* larvae may also affect wing size. Dutra et al. [49] found that under high larval density, *Wolbachia*-uninfected *Ae. aegypti* presented reduced wing size (centroid size) and a lower body glucose concentration.

Wing sizes are closely related to the flight capacity in insects. Individuals with longer wings are better at flying compared to those with shorter wings [14]. Long wings favour wider variation in speed and long flight duration [50]. We obtained longer wings in *S. calcitrans* reared in a group of five or when reared from donkey and sheep dung. Long wings allow insects to fly at a great speed for a long period of time and cover a large area [51, 52]. For instance, released *Ae. aegypti* with larger wings are more successful in host-seeking and oviposition site location [53–55]. We predict that *S. calcitrans* developed under lower density conditions are more likely to have good flight capacity. Also, developmental substrates such as donkey and sheep dung may increase the efficiency of *S. calcitrans* flight. This result supports Baleba et al. [39], who found that dung types preferred by female *S. calcitrans* for oviposition were best for offspring growth and development. In this case, preferred substrates lead to potential adult fitness benefits associated with wing size. Low larval density leads to larger adults with long wings due to higher resource availability for growth and nutrient storage in the larval stage. Similarly, development of larvae in preferred dung types would lead to larger adults with larger wings. Such changes may influence dispersal, mating, and vector competency of *S. calcitrans*, rendering this fly more capable of spreading pathogens.

Our study also showed that larval density and substrate type affected *S. calcitrans* wing shape. The thin-plate spline analyses showed that most of the shape changes (landmark movements) occurred on the radial (landmarks 11, 12, 13, 14 and 15) and medial (2, 3, 7, 8, 9 and 10) portions of the *S. calcitrans* wing. Oguz et al. [56] observed the same variation in the radial portion of the wing of *Phlebotomus tobbi* Adler & Theodore. Pieterse et al. [59] found variation in wing landmarks located at the costal, sub-costal and radial veins of *Bactrocera dorsalis* (Hendel) and *Ceratitis capitata* (Wiedemann) reared from nectarine, plum, pear and apple. According to Wootton et al. [57] and Shimmi et al. [58], the radial and the medial portions of insect wings play a critical function in the aerodynamics of insect flight. Wootton [60] suggested such changes may influence the wing strength, beat pattern and ultimately the dispersal potential of a fly. Therefore, the wing shape deformation observed here may affect the flight performance of *S. calcitrans*, their ability to find a host for a blood meal, and consequently, vectorial capacity. Several studies with no emphasis on wing morphology have already demonstrated the indirect effect of larval density and food quality on vector competence. For instance, in *Ae. albopictus*, a greater dissemination rate of Sindbis virus by the adult is the consequence of high levels of competition experienced by the larvae [61]. In *Anopheles stephensi* Liston, larvae developed in a nutritious substrate are more likely to transmit the human malaria parasite, *Plasmodium falciparum*, than those developed in a substrate with a poor nutritional value [62]. The discriminant factors on which the differentiation between flies reared from different density or dung type was based were not free of some allometric effects. In other words, wing size contributed significantly to wing shape variation. However, in the case of both larval density and developmental substrate, less than 3% of variation in wing shape was attributed to size. Such low residual variation indicates that changes in the relative position of landmarks as wing size increases are minimal [63].

Conclusions

This study highlights the effect of larval density and developmental substrate of wing size and wing shape of *S. calcitrans* using the landmark-based geometric morphometric method. The method satisfactorily discriminated *S. calcitrans* emerged from different larval densities and substrates based on the size and the shape of their wings. While there was a significant effect of size variation on variation in shape, but this accounted for less than 3% of variation. Future studies on flight performance of *S. calcitrans* as well as their vectorial capacity in pathogen transmission when reared under different larval conditions are required. However, our results demonstrate a role for larval density and developmental substrate to influence wing size and to some extent wing shape, which might have a significant effect on flight and dispersal of adult *S. calcitrans*.

Abbreviations

ANOVA: Analysis of variance; MANOVA: Multivariate analysis of variance; L: Light; D: Dark.

Acknowledgements

We thank Dr Joshua Njelembo Mbewe for the insightful advises and comments provided during the implementation of this work. We are also grateful to Pascal Atalor who generously assisted in the photography of wings.

Funding

We thank Deutscher Akademischer Austauschdienst (DAAD) for providing SBSB with a doctoral scholarship through the ARPPIS-DAAD scholarship programme. This work was supported by the IBCARP camel, grant no. DCI-FOOD/2014/ 346-739 - mainly by the European Union and Max Planck Institute for chemical ecology - *icipe* partner group to MNG. We also gratefully acknowledge the financial support for this research by the following organizations and agencies: Swedish International Development Cooperation Agency (Sida); UK Department for International Development (DFID); the Swiss Agency for Development and Cooperation (SDC); and the Kenyan Government. The views expressed herein do not necessarily reflect the official opinion of the donors.

Availability of data and materials

Data supporting the conclusions of this article are included within the article. All data collected during this study were archived in the Dryad data repository (https://doi.org/10.5061/dryad.37g5sk5)

Authors' contributions

SBSB: conceived the research idea, designed the experiment, collected and analysed the data, wrote the manuscript. DM: supervision and manuscript preparation. BT: supervision and manuscript preparation. CWW: supervision, contributed to experimental design, manuscript preparation and English editing. MNG: experimental design, supervision and manuscript preparation. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

Author details

[1] International Centre of Insect Physiology and Ecology (icipe), P.O. Box 30772-00100, Nairobi, Kenya. [2] Department of Zoology and Entomology, University of Pretoria, Private Bag X20, Hatfield 0028, South Africa.

Received: 16 February 2019 Accepted: 3 May 2019
Published online: 10 May 2019

References

1. Mousseau T. The adaptive significance of maternal effects. Trends Ecol Evol. 1998;13:403–7.
2. Heard SB. Imperfect oviposition decisions by the pitcher plant mosquito (*Wyeomyia smithii*). Evol Ecol. 1994;8:493–502.
3. Wong J, Morrison AC, Stoddard ST, Astete H, Chu YY, Baseer I, et al. Linking oviposition site choice to offspring fitness in *Aedes aegypti*: consequences for targeted larval control of dengue vectors. PLoS Negl Trop Dis. 2012;6:e1632.
4. Jirakanjanakit N, Dujardin J-P. Discrimination of *Aedes aegypti* (Diptera: Culicidae) laboratory lines based on wing geometry. Southeast Asian J Trop Med Public Health. 2005;36:4.
5. Wolf JB, Wade MJ. What are maternal effects (and what are they not)? Philos Trans R Soc Lond B Biol Sci. 2009;364:1107–15.
6. West-Eberhard MJ. Developmental plasticity and the origin of species differences. Proc Natl Acad Sci USA. 2005;102:6543–9.
7. Dogan M, Gunay F, Puggioli A, Balestrino F, Oncu C, Alten B, et al. Establishment of a satellite rearing facility to support the release of sterile *Aedes albopictus* males. I. Optimization of mass rearing parameters. Acta Trop. 2016;159:62–8.
8. Whitman DW, Agrawal AA. What is phenotypic plasticity and why is it important? In: Whitman DW, Ananthakrishnan TN, editors. Phenotypic plasticity of insects. Boca Raton: CRC Press; 2009. p. 1–63.
9. Johansson F, SöDerquist M, Bokma F. Insect wing shape evolution: independent effects of migratory and mate guarding flight on dragonfly wings: dragonfly wing shape evolution. Biol J Linn Soc. 2009;97:362–72.
10. Lin X, Xu Y, Jiang J, Lavine M, Lavine LC. Host quality induces phenotypic plasticity in a wing polyphenic insect. Proc Natl Acad Sci USA. 2018;115:7563–8.
11. Fraimout A, Jacquemart P, Villarroel B, Aponte DJ, Decamps T, Herrel A, et al. Phenotypic plasticity of *Drosophila suzukii* wing to developmental temperature: implications for flight. J Exp Biol. 2018;221:jeb166868.
12. Soto IM, Carreira VP, Soto EM, Hasson E. Wing morphology and fluctuating asymmetry depend on the host plant in cactophilic *Drosophila*. J Evol Biol. 2008;21:598–609.
13. Jong Z-W, Kassim NFA, Naziri MA, Webb CE. The effect of inbreeding and larval feeding regime on immature development of *Aedes albopictus*. J Vector Ecol. 2017;42:105–12.
14. DeVries PJ, Penz CM, Hill RI. Vertical distribution, flight behaviour and evolution of wing morphology in Morpho butterflies: wing evolution in Morpho butterflies. J Anim Ecol. 2010;79:1077–85.
15. Betts CR, Wootton RJ. Wing shape and flight behaviour in butterflies (Lepidoptera: Papilionoidea and Hesperioidea): a preliminary analysis. J Evol Biol. 1988;138:271–88.
16. Takken W, Klowden MJ, Chambers GM. Effect of body size on host seeking and blood meal utilization in *Anopheles gambiae* sensu stricto (Diptera: Culicidae): the disadvantage of being small. J Med Entomol. 1998;35:639–45.
17. Yoshioka M, Couret J, Kim F, McMillan J, Burkot TR, Dotson EM, et al. Diet and density dependent competition affect larval performance and oviposition site selection in the mosquito species *Aedes albopictus* (Diptera: Culicidae). Parasit Vectors. 2012;5:225.
18. Benelli G, Donati E, Romano D, Ragni G, Bonsignori G, Stefanini C, et al. Is bigger better? Male body size affects wing-borne courtship signals and mating success in the olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae): size and mating success in *B. oleae*. Insect Sci. 2016;23:869–80.

19. Trotta V, Duran Prieto J, Battaglia D, Fanti P. Plastic responses of some life history traits and cellular components of body size in *Aphidius ervi* as related to the age of its host *Acyrthosiphon pisum*: wing shape variation in *A. ervi*. Biol J Linn Soc. 2014;113:439–54.
20. Motoki MT, Suesdek L, Bergo ES, Sallum MAM. Wing geometry of *Anopheles darlingi* Root (Diptera: Culicidae) in five major Brazilian ecoregions. Infect Genet Evol. 2012;12:1246–52.
21. Gómez GF, Márquez EJ, Gutiérrez LA, Conn JE, Correa MM. Geometric morphometric analysis of Colombian *Anopheles albimanus* (Diptera: Culicidae) reveals significant effect of environmental factors on wing traits and presence of a metapopulation. Acta Trop. 2014;135:75–85.
22. Changbunjong T, Sumruayphol S, Weluwanarak T, Ruangsittichai J, Dujardin J-P. Landmark and outline-based geometric morphometrics analysis of three *Stomoxys* flies (Diptera: Muscidae). Folia Parasitol. 2016;63:037.
23. Wilke ABB, Christe R, Multini LC, Vidal PO, Wilk-da-Silva R, de Carvalho GC, et al. Morphometric wing characters as a tool for mosquito identification. PLoS ONE. 2016;11:0161643.
24. Vidal PO, Suesdek L. Comparison of wing geometry data and genetic data for assessing the population structure of *Aedes aegypti*. Infect Genet Evol. 2012;12:591–6.
25. Virginio F, Oliveira Vidal P, Suesdek L. Wing sexual dimorphism of pathogen-vector culicids. Parasit Vectors. 2015;8:159.
26. Yeap H, Axford JK, Popovici J, Endersby NM, Iturbe-Ormaetxe I, Ritchie SA, et al. Assessing quality of life-shortening *Wolbachia*-infected *Aedes aegypti* mosquitoes in the field based on capture rates and morphometric assessments. Parasit Vectors. 2014;7:58.
27. Sendaydiego JP, Demayo CG. Describing variations in wing shapes of *Anopheles flavirostris* detected positive and negative of filaria using relative warp and Euclidean distance matrix analysis. Int J Mosq Res. 2015;2:09–13.
28. Kitthawee S, Rungsri N. Differentiation in wing shape in the *Bactrocera tau* (Walker) complex on a single fruit species of Thailand. Scienceasia. 2011;37:308–13.
29. Sandoval Ramirez CM, Nieves Blanco EE, Gutiérrez Marin R, Jaimes Mendez DA, Rodríguez NO, Otálora-Luna F, et al. Morphometric analysis of the host effect on phenotypical variation of *Belminus ferroae* (Hemiptera: Triatominae). J Entomol. 2015;2015:1–12.
30. Alves VM, Moura MO, de Carvalho CJB. Wing shape is influenced by environmental variability in *Polietina orbitalis* (Stein) (Diptera: Muscidae). Rev Bras Entomol. 2016;60:150–6.
31. Viscosi V, Cardini A. Leaf morphology, taxonomy and geometric morphometrics: a simplified protocol for beginners. PLoS ONE. 2011;6:e25630.
32. Lorenz C, Almeida F, Almeida-Lopes F, Louise C, Pereira SN, Petersen V, et al. Geometric morphometrics in mosquitoes: what has been measured? Infect Genet Evol. 2017;54:205–15.
33. Lehane MJ. The biology of blood-sucking in insects. New York: Cambridge University Press; 2005.
34. Pitzer JB, Kaufman PE, Tenbroeck SH, Maruniak JE. Host blood meal identification by multiplex polymerase chain reaction for dispersal evidence of stable flies (Diptera: Muscidae) between livestock facilities. J Med Entomol. 2011;48:53–60.
35. Baldacchino F, Muenworn V, Desquesnes M, Desoli F, Charoenviriyaphap T, Duvallet G. Transmission of pathogens by *Stomoxys* flies (Diptera, Muscidae): a review. Parasite. 2013;20:26.
36. Carn VM. The role of dipterous insects in the mechanical transmission of animal viruses. BVA. 1996;152:377–93.
37. Walker AR. Disease caused by arthropods. In: Sewell MMH, Brocklesby DW, editors. Handbook on animal diseases in the tropics. 4th ed. London: Bailliere Tindall; 1990. p. 1–385.
38. Taylor DB, Moon RD, Mark DR. Economic impact of stable flies (Diptera: Muscidae) on dairy and beef cattle production. J Med Entomol. 2012;49:198–209.
39. Baleba SBS, Torto B, Masiga D, Weldon CW, Getahun MN. Egg-laying decisions based on olfactory cues enhance offspring fitness in *Stomoxys calcitrans* L (Diptera: Muscidae). Sci Rep. 2019;9:3850.
40. Suesdek L, Lorenz C. Evaluation of chemical preparation on insect wing shape for geometric morphometrics. Am J Trop Med Hyg. 2013;89:928–31.
41. Schneider CA, Rasband WS, Eliceiri KW. NIH image to ImageJ: 25 years of image analysis. Nat Methods. 2012;9:671–5.
42. Klingenberg CP, Barluenga M, Meyer A. Shape analysis of symmetric structures: quantifying variation among individuals and asymmetry. Evolution. 2002;56:1909–20.
43. Hammer O, Harper DAT, Ryan PD. PAST: Paleontological Statistics Software: package for education and data analysis. Palaeontol Electron. 2001;4:4–9.
44. Ribak G, Barkan S, Soroker V. The aerodynamics of flight in an insect flight-mill. PLoS One. 2017;12:e0186441.
45. R Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org/.2018.
46. Klingenberg CP. MorphoJ: an integrated software package for geometric morphometrics: computer program note. Mol Ecol Resour. 2011;11:353–7.
47. Jirakanjanakit N, Dujardin J-P. Discrimination of *Aedes aegypti* (Diptera: Culicidae) laboratory lines based on wing geometry. Southeast Asian J Trop Med Public Health. 2005;36:4.
48. Stephens CR, Juliano SA. Wing shape as an indicator of larval rearing conditions for *Aedes albopictus* and *Aedes aegypti* (Diptera: Culicidae). J Med Entomol. 2012;49:927–38.
49. Yeap HL, Hoffmann AA, Ross PA, Endersby NM. Larval competition extends developmental time and decreases adult size of wMelPop *Wolbachia*-infected *Aedes aegypti*. Am J Trop Med Hyg. 2014;91:198–205.
50. Dutra HLC, Lopes da Silva V, da Rocha Fernandes M, Logullo C, Maciel-de-Freitas R, Moreira LA. The influence of larval competition on Brazilian *Wolbachia*-infected *Aedes aegypti* mosquitoes. Parasit Vectors. 2016;9:282.
51. Hoffmann AA, Ratna E, Sgrò CM, Barton M, Blacket M, Hallas R, et al. Antagonistic selection between adult thorax and wing size in field released *Drosophila melanogaster* independent of thermal conditions. J Evol Biol. 2007;20:2219–27.
52. Berwaerts K, Dyck HV, Aerts P. Does flight morphology relate to flight performance? An experimental test with the butterfly *Pararge aegeria*. Funct Ecol. 2002;16:484–91.
53. Davis AK, Holden MT. Measuring intraspecific variation in flight-related morphology of monarch butterflies (*Danaus plexippus*): which sex has the best flying gear? J Insects. 2015;2015:1–6.
54. Kay BH, Muir LE. *Aedes aegypti* survival and dispersal estimated by mark-release-recapture in northern Australia. Am J Trop Med Hyg. 1998;58:277–82.
55. Harrington LC, Scott TW, Lerdthusnee K, Coleman RC, Costero A, Clark GG, et al. Dispersal of the dengue vector *Aedes aegypti* within and between rural communities. Am J Trop Med Hyg. 2005;72:209–20.
56. Oguz G, Kasap OE, Alten B. Wing morphology variations in a natural population of *Phlebotomus tobbi* Adler and Theodor 1930. J Vector Ecol. 2017;42:223–32.
57. Wootton RJ, Herbert RC, Young PG, Evans KE. Approaches to the structural modelling of insect wings. Philos Trans R Soc Lond Biol Sci B. 2003;358:1577–87.
58. Shimmi O, Matsuda S, Hatakeyama M. Insights into the molecular mechanisms underlying diversified wing venation among insects. Philos Trans R Soc Lond Biol Sci B. 2014;281:20140264.
59. Pieterse W, Benítez HA, Addison P. The use of geometric morphometric analysis to illustrate the shape change induced by different fruit hosts on the wing shape of *Bactrocera dorsalis* and *Ceratitis capitata* (Diptera: Tephritidae). Zool Anz. 2017;269:110–6.
60. Wootton RJ. Support and deformability in insect wings. J Zool. 1981;193:447–68.
61. Alto BW, Lounibos LP, Higgs S, Juliano SA. Larval competition differentially affects arbovirus infection in *Aedes* mosquitoes. Ecology. 2005;86:3279–88.
62. Shapiro LLM, Murdock CC, Jacobs GR, Thomas RJ, Thomas MB. Larval food quantity affects the capacity of adult mosquitoes to transmit human malaria. Proc R Soc Biol Sci B. 2016;283:20160298.
63. Dujardin J-P. Morphometrics applied to medical entomology. Infect Genet Evol. 2008;8:875–90.

Ecological Entomology

Ecological Entomology (2019), DOI: 10.1111/een.12748

Larval experience of stable fly, *Stomoxys calcitrans* Linneaus, 1758 (Diptera: Muscidae) does not influence oviposition preference in gravid females

STEVE B.S. BALEBA,[1,2] CHRISTOPHER W. WELDON,[2] DANIEL MASIGA,[1] BALDWYN TORTO[1,2] and MERID N. GETAHUN[1] [1]International Centre of Insect Physiology and Ecology (icipe), Nairobi, Kenya and [2]Department of Zoology and Entomology, University of Pretoria, Hatfield, South Africa

Abstract. 1. Hopkins' host selection principle (HHSP) proposes that, in holometabolous insects, gravid females prefer to oviposit on their developmental substrate. This hypothesis is widely explored in phytophagous insects, but few studies have considered blood-feeding insects. In this study, the HHSP was tested using the haematophagous stable fly, *Stomoxys calcitrans* (Diptera: Muscidae). Using no-choice and two-choice tests, the study first sought to demonstrate whether *S. calcitrans* larvae recognise their rearing substrate.

2. It was found that when a rearing substrate is offered to *S. calcitrans* larvae singly (no-choice) or associated with a non-rearing substrate (two-choice), they were able to recognise the substrate in which they developed. This ability disappeared when larvae were transferred to another substrate for 5 h.

3. Next, using oviposition bioassays, it was investigated whether information gathered by pre-imaginal stages during their development can persist into the adult stages after metamorphosis. It was hypothesised that gravid females emerging from clean and uncleaned pupae reared on sheep and camel dung would still prefer to lay on their rearing substrate. The results revealed that gravid females did not prefer to oviposit on their developmental substrate. In addition, failure to recognise and prefer their developmental substrate was found in gravid females that had emerged from puparia that were cleaned as well as those contaminated with sheep dung.

4. It is concluded that *S. calcitrans* adult preference is not influenced by their offspring's experience. Overall, the HHSP was not supported by our study, suggesting that this principle may not apply to generalist insects characterised by high plasticity in oviposition substrate acceptance.

Key words. Hopkins' host selection principle, larvae experience, oviposition, *Stomoxys calcitrans*.

Introduction

In holometabolous insects, pre-imaginal stages (egg and larva) and the adult stage are different in terms of their body form, life style, and diet. However, it may be possible that information gathered by pre-imaginal stages is retained during metamophosis to be transferred to the adult. Several studies have claimed a direct or indirect effect of larval experience on adult behaviour in different holometabolous groups such as Lepidoptera (Olsson *et al.*, 2006; Proffit *et al.*, 2015; Hu *et al.*, 2018), Diptera (Tully *et al.*, 1994; Ray, 1999; Blackiston *et al.*, 2008), Coleoptera (Reh-Hamburg, 1924; Rietdorf & Steidle, 2002), and Hymenoptera (Lecomte & Thibout, 1993; Gandolfi *et al.*, 2003), although others show no support for a connection (Rojas & Wyatt, 1999; Kerpel & Moreira, 2005; Janz *et al.*, 2008). Three hypotheses have been raised to provide a functional mechanism for information retention across life stages: 'Hopkins' host selection principle' (Hopkins, 1916), the 'neo-Hopkins principle' (Jaenike, 1983) and the 'chemical legacy hypothesis'

Correspondence: Steve B.S. Baleba, International Centre of Insect Physiology and Ecology (icipe), P.O. Box 30772-00100, Nairobi, Kenya. E-mail: bbaleba@icipe.org

(Corbet, 1985). Hopkins' host selection principle (HHSP) refers to the observation that, in insects, adult stage behaviour is conditioned by experience during their pre-imaginal stages. For the neo-Hopkins principle, adult behaviour is shaped by the experience gained during or shortly after emergence from pupae. The chemical legacy hypothesis considers that the connection between larval and adult behaviour is made by the chemical cues bequeathed by the larval stage to the adult stage. In practice, when illustrating the link between larval and adult stage memories, it is necessary to clean and physically separate pupae from chemical cues associated with the larval environment to eliminate the opportunity for habituation or sensitisation of the emerging adults (Tully *et al.*, 1994; Corbet, 1985; Rietdorf & Steidle, 2002; Gandolfi *et al.*, 2003). By doing so, a chemical legacy can be ruled out. Thereafter, any connection between larval and adult experience would result from the transmission of neuronal information from the larva to the adult during metamorphosis, enabling persistence in the adult brain of memories formed during the larval stage (Blackiston *et al.*, 2008). Leimar *et al.* (2006) suggested that biological functions such as oviposition rely on genetic and environmental cues and that organisms will tend to use the cues that can most accurately predict the conditions that organisms will encounter. Under most conditions, genetic cues will be favoured due to their higher efficiency, but environmental cues can be important under some conditions. In general, it is also postulated that gravid females of insects are genetically programmed to oviposit on substrates that guarantee the fitness of their offspring (Jaenike, 1978; Thompson 1988). Along these lines, Janz *et al.* (2008) argued that in conditions where substrate suitability is unpredictable, selection of appropriate substrate by gravid females should be governed by genetic cues. On the other hand, if temporal variation in substrate suitability is high but predictable, gravid females may use larval cues, especially if it is costly for adult females to gather information on substrate availability and suitability. It is therefore expected that the HHSP will be more beneficial in environments lacking alternative oviposition substrates but where availability can shift with time, e.g. due to agricultural practices.

The effect of pre-imaginal conditioning in adult behaviour is well documented in phytophagous insects. Tully *et al.* (1994), after electroshocking larvae of *Drosophila* in the presence of ethyl acetate or isoamyl acetate, found that the conditional odour avoidance observed in larvae was still present in adults 8 days later. In *Musca domestica*, Ray (1999) showed that adults preferred specific odours to which they were exposed during larval development. Host selection behaviour in adult granary weevils, *Sitophilus granarius*, was shaped by their experience during the immature stages (Rietdorf & Steidle, 2002). The onion fly, *Delia antiqua*, preferred to oviposit on their natal host (Ning *et al.*, 2018). In haematophagous insects (mainly in mosquitoes), learning in the larval (Ferrari *et al.*, 2008; Baglan *et al.*, 2017) and adult stages (Menda *et al.*, 2013; Roberts, 2014) has already been demonstrated. However, little is known about the possible transmission of information from the larva to adult in haematophagous insects. To our knowledge, only McCall and Eaton (2001) explored this, by showing that females of *Culex quinquefasciatus* reared in water containing skatole (3-methylindole) preferred to oviposit on water containing that compound. Therefore, in this study, we examine whether the information gathered by larvae of a blood-feeding insect can reach the adult stage through metamorphosis, using the stable fly, *Stomoxys calcitrans*, as a model.

Stomoxys calcitrans is a cosmopolitan blood-feeding insect that mechanically transmits viruses (e.g. West Nile fever virus, Rift Valley fever virus), bacteria (e.g. *Bacillus anthracis*, *Pasteurella multocida*), protozoans (e.g. *Trypanosoma evansi*, *Besnoitia besnoit*), and helminths (e.g. *Habronema microstoma*, *Dirofilaria repens*) to their adult hosts, which include cattle, camels, horses, dogs, and humans (Lehane, 2005; Pitzer *et al.*, 2011; Baldacchino *et al.*, 2013). During outbreaks, *S. calcitrans* can reduce weight gain in cattle by up to 19%, and lead to a 40–60% reduction in milk yield (Carn, 1996; Walker, 1990). In the U.S.A., Taylor *et al.* (2012) estimated economic losses attributable to *S. calcitrans* infestation at around $2.2 billion year^{-1}. Like all dipteran species, *S. calcitrans* has a holometabolous life cycle. Eggs are laid and the larval stage (with three instars) and pupal stage are completed in the dung of herbivorous animals such as cows, donkeys, horses, goats, and sheep (Hafez & Gamal-Eddin, 1959; Jeanbourquin & Guerin 2007; Cançado et *al.*, 2013). Baleba *et al.* (2019) demonstrated that gravid females of *S. calcitrans* preferred to oviposit on donkey and sheep dung over camel and donkey, which led to greater fitness in pre-imaginal stages. In this study, we investigated whether this preference–performance behaviour was fixed or whether it was influenced by experience of *S. calcitrans* larvae gained throughout their development. Specifically, we determined the ability of *S. calcitrans* larvae to recognise their rearing substrate and established whether recognition of the rearing substrate was maintained for 5 h. Finally, we assessed whether rearing substrate recognition persisted in gravid females. Our results are important in the use of dung chemical cues as attractants in surveillance and management of *S. calcitrans*.

Materials and methods

Test insects

Stomoxys calcitrans flies were obtained from a culture established at the Duduville campus of the International Centre of Insect Physiology and Ecology (*icipe*) in Nairobi (1°13′12″S, 36°52′48″ E; *c.* 1600 m above sea level). Adults reared from rabbit dung were kept in cages (75 × 60 × 45 cm) under buffered conditions of 25 ± 5 °C, 65 ± 5% RH and LD 12:12 h. Flies were fed twice per day (08.00 and 16.00 hours) on defibrinated bovine blood (from slaughter houses) poured on moistened cotton. When adults were sexually mature, camel and sheep dung placed in the plastic container (21.5 × 14.5 × 7.4 cm) were presented to gravid females for oviposition. After a period of 24 h, each substrate was removed and transferred to another cage. Larval development was monitored daily until the third larval instar (L3) was reached. This developmental stage was identified by inspecting the posterior spiracles, which are characterised by triangular discs with three sinuous slits (Friesen *et al.*, 2017).

Experience test

To establish whether *S. calcitrans* larvae change their behaviour in relation to experience, we hypothesised that L3 larvae of *S. calcitrans* reared on a specific substrate would be attracted to the same substrate. To test this hypothesis, we began with a 'no-choice' test (Fig. 1a) where L3 larvae reared on camel dung (Fig. 1a,i) or sheep dung (Fig. 1a,ii) were gently placed away from either substrate in an arena (described later) using soft forceps. This was done to confirm that larvae were capable of relocating the substrate within an arena. Afterwards, we performed a series of 'two-choice' tests. For the first two-choice test (Fig. 1b), L3 larvae reared on camel dung (Fig. 1b,i) or sheep dung (Fig. 1b,ii) were permitted to choose between camel and sheep dung in an arena. We established that L3 larvae preferred their rearing substrate over non-rearing substrate. To elucidate whether this behaviour was the result of innate preference for particular dung types, we hypothesised that if L3 larvae were presented with two unfamiliar substrates, they would exhibit no preference for either substrate. To test this, we reared L3 larvae on camel dung and exposed them to rabbit and sheep dung (Fig. 1c,i). We also reared L3 larvae on sheep dung and exposed them to camel and rabbit dung (Fig. 1c,ii).

For all tests, we used plastic containers ($21.5 \times 14.5 \times 7.4$ cm) as a choice arena in which 80 larvae were released one by one in the middle of the container (7.5 cm away from the tested substrates). The amount of substrate in each test arena was 50 g. At 10 min after setting up each replicate, larvae found on a substrate were considered to have made a choice. The duration of the tests was selected based on the results of preliminary bioassays where larvae were given 5, 10, 15, and 20 min to move to a substrate. The number of larvae moving to a particular substrate did not improve significantly [10 min, 57 (71.25%); 20 min, 62 (77.5%); $n = 80$, $\chi^2 = 0.21$, d.f = 1, $P = 0.65$] if larvae were given longer than 10 min (data not presented). Larvae that were still in the middle of the container were recorded as not having made a choice. The substrate position was changed between replicates. All tests were conducted in darkness because *S. calcitrans* larvae exhibit negative phototaxis. After each test, containers were cleaned with distilled water and tissue paper to avoid retention of odour from dung. We did not use organic solvents because they could be toxic to the larvae or represented a source of contamination. During all the bioassays, we used camel and sheep dung as rearing substrate based on the difference in their physicochemical properties (Baleba et *al*., 2019)

Experience duration test

In the previous bioassays, we showed that *S. calcitrans* larvae preferred their substrate of development. Here we sought to determine how long this preference was retained. We hypothesised that L3 larvae would progressively lose their aptitude to recognise their rearing substrate when staying in another substrate. To test this, we transferred L3 larvae reared on camel dung to sheep dung for 1 h (Fig. 2a,i), 5 h (Fig. 2b,i), and 24 h (Fig. 2c,i). Thereafter, we allowed individuals from each treatment to choose between camel and sheep dung in two-choice tests (as described earlier), to see if they still preferred their rearing substrate. We did the same for L3 larvae reared on sheep dung by transferring them to camel dung for 1 h (Fig. 2a,ii), 5 h (Fig. 2b,ii), and 24 h (Fig. 2c,ii). We selected the different novel dung exposure times based on the review by Margulies *et al.* (2005), which differentiated among three types of memory in *Drosophila*: short- (1 h), middle- (5 h) and long-term (24 h) memory.

Experience recovery test

The preceding experiment revealed that sojourning in another dung type for 5 and 24 h led to loss of *S. calcitrans* larval preference for the initial rearing substrate. We aimed to test if this loss of preference could be recovered. To do so, we transferred L3 larvae reared on camel dung to sheep dung for 24 h, then returned them to camel dung for a further 24 h (Fig. 3a). After returning larvae to their original substrate, we allowed them to choose between camel and sheep dung in two-choice tests. Again, the reciprocal procedure was applied to L3 larvae reared on sheep dung (Fig. 3b). As in the previous experiment, 80 L3 larvae were released individually in the middle of the arena, and their position was recorded after 10 min exposure in darkness.

Hopkins' host selection principle and chemical legacy hypothesis tests

Hopkins' host selection principle test. We demonstrated that *S. calcitrans* larvae recognise their rearing substrate. According to the HHSP, this experience should persist in *S. calcitrans* adults. Therefore, we predicted that gravid females of *S. calcitrans* reared on sheep or camel dung would still prefer to lay on their rearing substrate. To test this, we reared *S. calcitrans* separately on sheep and camel dung, but we treated their pupae in two ways. In the first treatment (Fig. 4a), we left pupae in contact with their rearing substrate so that emerging adults were exposed to the chemical cues from the substrate. For the second treatment (Fig. 4b), we prevented contact of emerging adults with their substrate following the procedure described by Liu and Liu (2006). Briefly, we removed pupae from the substrate, gently cleaned them with distilled water and tissue paper, and transferred them to a sterilised cage ($34 \times 34 \times 34$ cm) for adult emergence. Once emerged, adults were fed with defibrinated bovine blood poured on wet cotton and kept on this feeding regime until females were gravid (Fig. 4a,i, b,i). The obtained gravid females were used in the oviposition tests (Fig. 4a,ii, b,ii) as follows. Ten gravid females were transferred to a cage ($34 \times 34 \times 34$ cm) before being presented with 30 g of oviposition substrate in a Petri dish (diameter 5.5 cm). Gravid females from sheep dung were presented with donkey and sheep dung, and gravid females from camel dung where presented with camel and sheep dung. The number of eggs deposited on each substrate was counted after 24 h. Tests using each treatment were replicated 10 times and a new group of flies was used in each replicate.

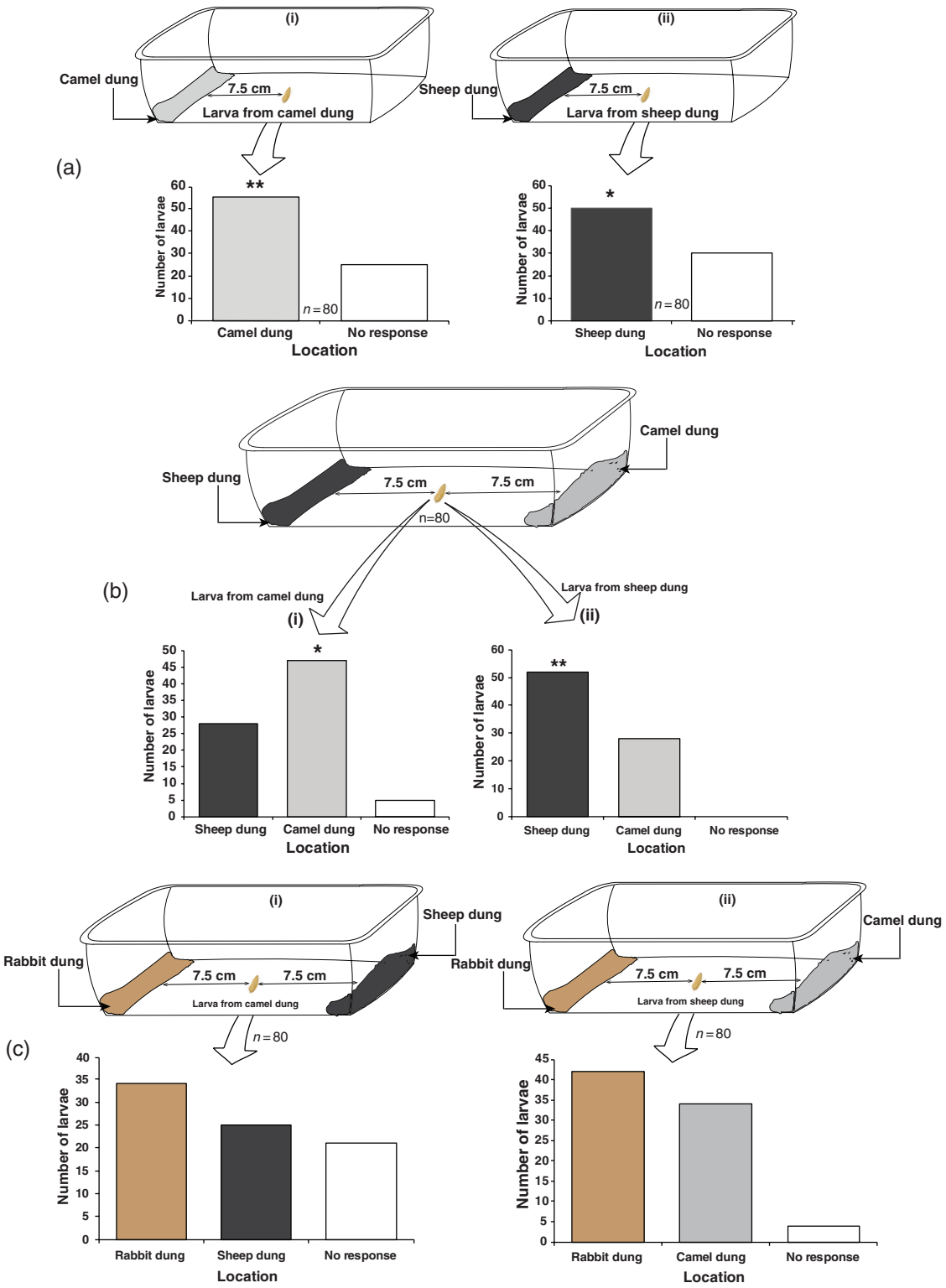

Fig. 1. *Stomoxys calcitrans* larvae recognise the substrate in which they developed. (a) No-choice test with third-instar (L3) larvae reared on camel dung (i) or sheep dung (ii) ($n = 80$). (b) Two-choice test with reared and non-reared substrates of camel and sheep dung: L3 larvae reared on camel dung (i) or sheep dung (ii) ($n = 80$). (c) Two-choice test with two non-reared substrates: (i) L3 larvae from camel dung given a choice between rabbit and sheep dung; (ii) L3 larvae from sheep dung given a choice between rabbit and camel dung. Asterisks indicate proportions that differed significantly from random (50%) determined by a two-tailed binomial test: $*P < 0.05$; $**P < 0.01$. [Colour figure can be viewed at wileyonlinelibrary.com].

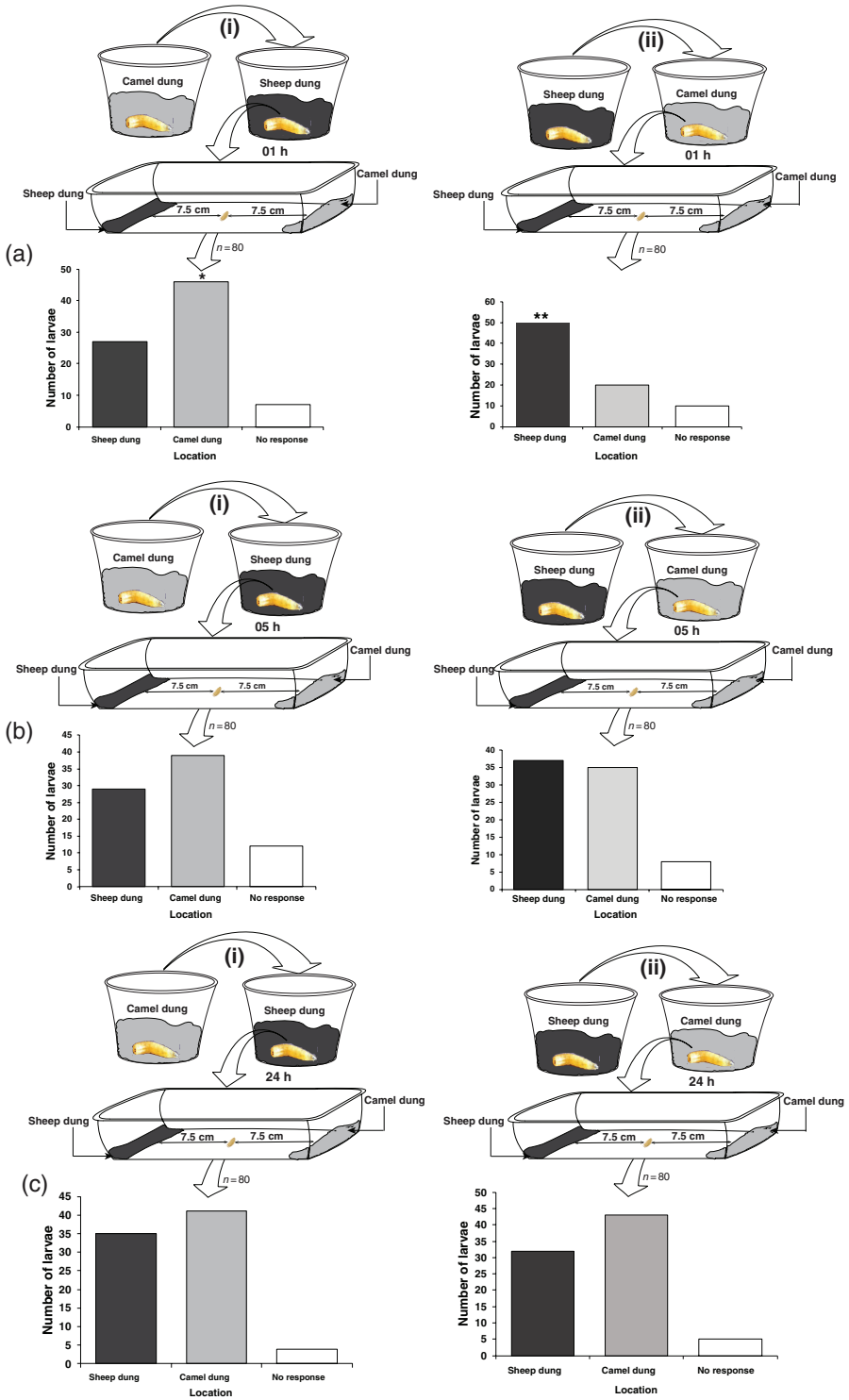

Fig. 2. *Stomoxys calcitrans* larval experience in locating their rearing substrate lasts for a short period (1 h). (a) Experience test with larvae left in a non-rearing substrate for 1 h: (i) bioassay with third-instar (L3) larvae reared on camel dung; (ii) bioassay with L3 larvae reared on sheep dung. (b) Experience test with larvae left in a non-rearing substrate for 5 h: (i) bioassay with L3 larvae reared on camel dung; (ii) bioassay with L3 larvae reared on sheep dung. (c) Experience test with larvae left in a non-rearing substrate for 24 h: (i) bioassay with L3 larvae reared on camel dung; (ii) bioassay with L3 larvae reared on sheep dung. Asterisks indicate proportions that differed significantly from random (50%) determined by a two-tailed binomial test: $*P < 0.05$; $**P < 0.01$. The absence of asterisks indicates no significant preference by larvae for the substrates provided (two-tailed binomial test, $P > 0.05$). [Colour figure can be viewed at wileyonlinelibrary.com].

 Ecological Entomology, doi: 10.1111/een.12748

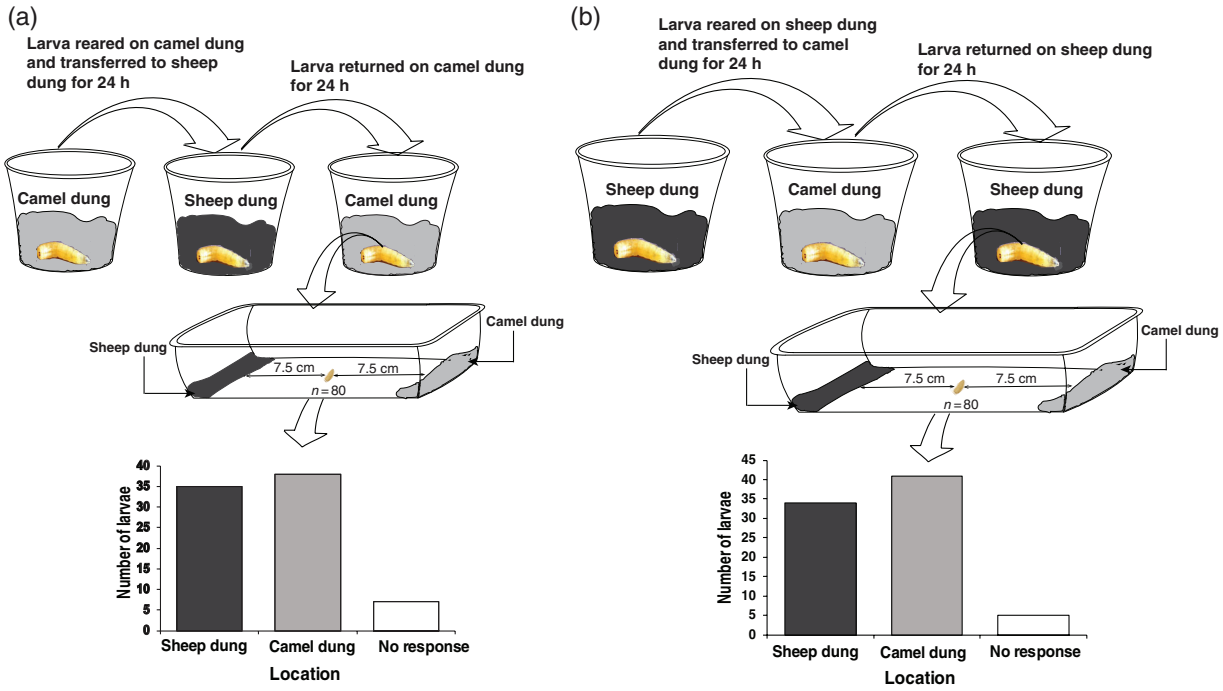

Fig. 3. *Stomoxys calcitrans* larval experience is not recoverable. (a) Memory recovery test with larvae reared on camel dung; (b) memory recovery test with larvae reared on sheep dung. In the two tests, there was no significant preference by larvae for the substrates provided (two-tailed binomial test, $P > 0.05$). [Colour figure can be viewed at wileyonlinelibrary.com].

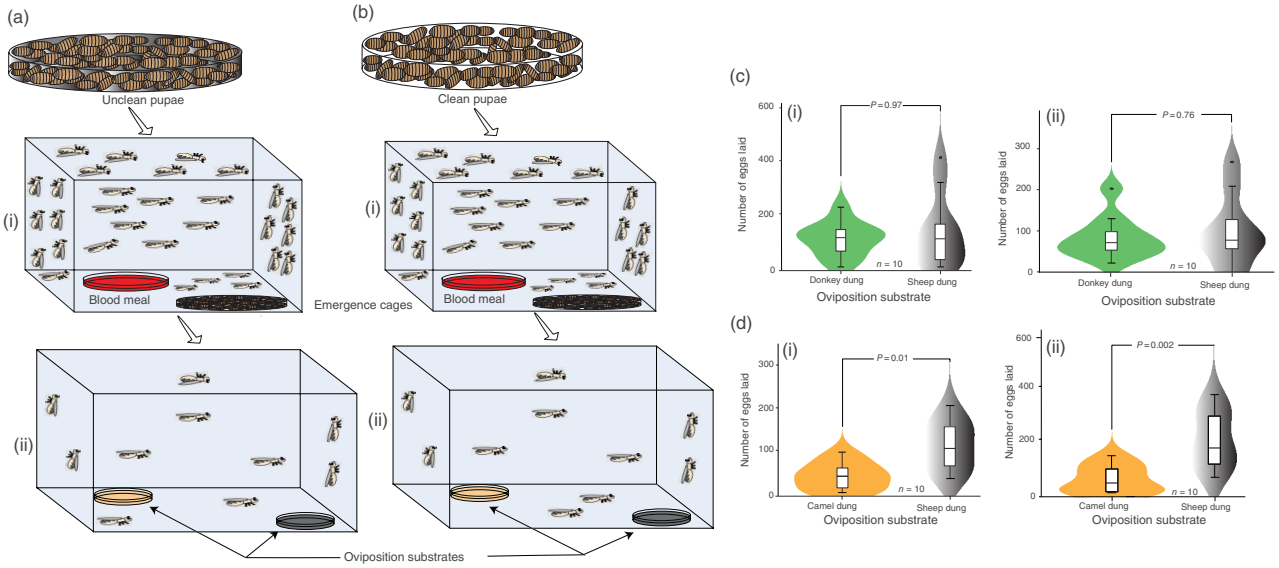

Fig. 4. Oviposition behaviour in gravid females of *Stomoxys calcitrans* is not affected by the larval experience. (a) Oviposition bioassay using *S. calcitrans* gravid females from unclean pupae: (i) unclean pupae, adult's emergence cage; (ii) oviposition test arena. (b) Oviposition bioassay using *S. calcitrans* gravid females from cleaned pupae: (i) cleaned pupae, adult's emergence cage; (ii) oviposition test arena. (c) Violin plots showing no significant difference (Mann–Whitney test, $P > 0.05$) in number of eggs laid on donkey and sheep dung by gravid females reared on sheep dung and emerged from cleaned pupae (i) and uncleaned pupae (ii). (d) Violin plots showing significant difference (Mann–Whitney test, $P < 0.05$) in number of eggs laid on camel and sheep dung by gravid females reared on camel dung and emerged from clean pupae (i) and unclean pupae (ii). The bars in each box show the median and those at the extremity of the box show the 25–75th percentiles, which are extended by whiskers indicating 1.5× interquartile range from the 25th to 75th percentiles; the shape denotes the density estimate and extends to extreme values. [Colour figure can be viewed at wileyonlinelibrary.com].

Chemical legacy hypothesis test. According to this hypothesis, the traces of chemical cues on the surface of pupae could influence adult oviposition substrate preference. To test this, we reared *S. calcitrans* on rabbit dung until the pupal stage (Fig. 5a,b) and we divided the obtained pupae into two groups. We maintained the first group of pupae on rabbit dung until adult emergence (Fig. 5a,i), while we cleaned the second group of pupae as described earlier and transferred them to sheep dung for contamination until adult emergence (Fig. 5b,i). Once emerged, we fed adults with defibrinated bovine blood. Ten gravid females were used in the oviposition bioassay (Fig. 5a,ii, b,ii) with a choice of rabbit and sheep dung provided to them in separate Petri dishes. The number of eggs laid on each substrate was counted after 24 h. Each test was replicated 10 times and a new group of flies was used in each replicate.

Data analysis

All statistical analyses were performed using R 3.5.0 (R Core Team, 2018). For the no-choice test, we compared the proportion of larvae found in dung with those that were unable to join the dung after 10 min exposure. For choice tests, we compared the proportion of larvae that had chosen between two types of exposed dung. In both no-choice and choice tests, we used the two-tailed binomial test (McDonald, 2009). For the oviposition bioassay, as the collected data were not normally distributed, we used the non-parametric Wilcoxon–Mann–Whitney test to compare the number of eggs laid on different substrates (Dalgaard, 2008).

All data collected during this study are archived in the Dryad data repository.

Results

Experience test

In the 'no-choice' test, where 80 larvae were placed individually away from their rearing substrate (Fig. 1a), a significant majority of larvae reared from camel dung (Fig. 1a,i) returned to it (binomial test: 68.8% success, 31.2% failure, $P = 0.001$). Likewise, a significant majority of larvae from sheep dung (Fig. 1a,ii) found their rearing substrate (binomial test: 62.5% success, 37.5% failure, $P = 0.033$). The remaining larvae from camel (25 larvae) and sheep (30 larvae) dung did not reach their substrate after 10 min.

For the first 'two-choice' test (Fig. 1b), when offering a choice to 80 larvae between their rearing substrate and a non-rearing substrate, we found that in larvae reared on camel dung (Fig. 1b,i), five larvae did not make a choice (6.35% non-response). For the 75 remaining larvae, significantly more selected their rearing substrate (camel dung) and the remainder went to sheep dung (binomial test: 62.7% success, 37.3% failure, $P = 0.037$). For the larvae reared on sheep dung (Fig. 1b,ii), all 80 tested larvae made a choice (0% non-response). We found that a significant majority selected sheep dung over camel dung (binomial test: 65% success, 35% failure, $P = 0.010$).

For the second 'two-choice test' bioassay (Fig. 1c) where we provided larvae with a choice between two non-rearing substrates, they did not exhibit any preference. Among the 80 larvae reared on camel dung and offered rabbit or sheep dung (Fig. 1c,i), of those that reached either substrate, the proportion of larvae in rabbit (42.5%) or sheep dung (31.3%) did not differ from random (binomial test, $P = 0.141$). Also, for the larvae reared on sheep dung (Fig. 1c,ii), the proportion of larvae on rabbit dung (52.5%) or camel dung (42.5%) did not differ significantly (binomial test, $P = 0.26$).

Experience duration test

Results from the experience duration test show that the ability of *S. calcitrans* larvae to recognise their rearing substrate lasts for up to 1 h. Larvae reared on camel dung and transferred to sheep dung for 1 h recognised their rearing substrate (binomial calculation: 63.01% success, 36.99% success, $P = 0.034$; Fig. 2a,i). Similar results were found for larvae reared on sheep dung and transferred to camel dung for 1 h (binomial calculation: 71.4% success, 28.6% success, $P < 0.001$; Fig. 2a,ii). After sojourning on another substrate for 5 h, larvae reared on camel dung (binomial calculation: 55.4% success, 44.6% failure, $P = 0.275$; Fig. 2b,i) and sheep dung (binomial calculation: 55.4% success, 48.6% failure, $P = 0.910$; Fig. 2b,ii) did not recognise their rearing substrate. This loss of experience was also evident in larvae that spent 24 h in another substrate. Larvae reared on camel dung after sojourning on sheep dung for 24 h did not discriminate between camel or sheep dung (binomial calculation: 53.9% success, 46.1% failure, $P = 0.417$; Fig. 2c,i). Similarly, larvae from sheep dung that spent 24 h on donkey dung did not differentiate between sheep or camel dung (binomial calculation: 42.7% success, 57.3% failure, $P = 0.103$; Fig. 2c,ii).

Experience recovery test

In the experience recovery test, we found that *S. calcitrans* larvae, after being maintained in a novel substrate for 24 h and transferred back to their original rearing substrate for 24 h, did not recover a preference for their substrate of provenance. Larvae reared on camel dung, transferred to sheep dung, then put back on camel dung did not significantly prefer camel dung as predicted (binomial calculation: 52.05% success, 47.95% failure, $P = 0.741$; Fig. 3a). Also, larvae reared on sheep dung, transferred to camel dung and returned to sheep dung did not prefer their original rearing substrate (binomial calculation: 45.3% success, 54.7% failure, $P = 0.327$; Fig. 3b).

Hopkins' host selection principle and chemical legacy hypothesis tests

Hopkins' host selection principle test. According to the result of our oviposition bioassays, *S. calcitrans* gravid females reared on sheep and camel dung did not lay more eggs on sheep and camel dung, respectively, as predicted by the HHSP. The

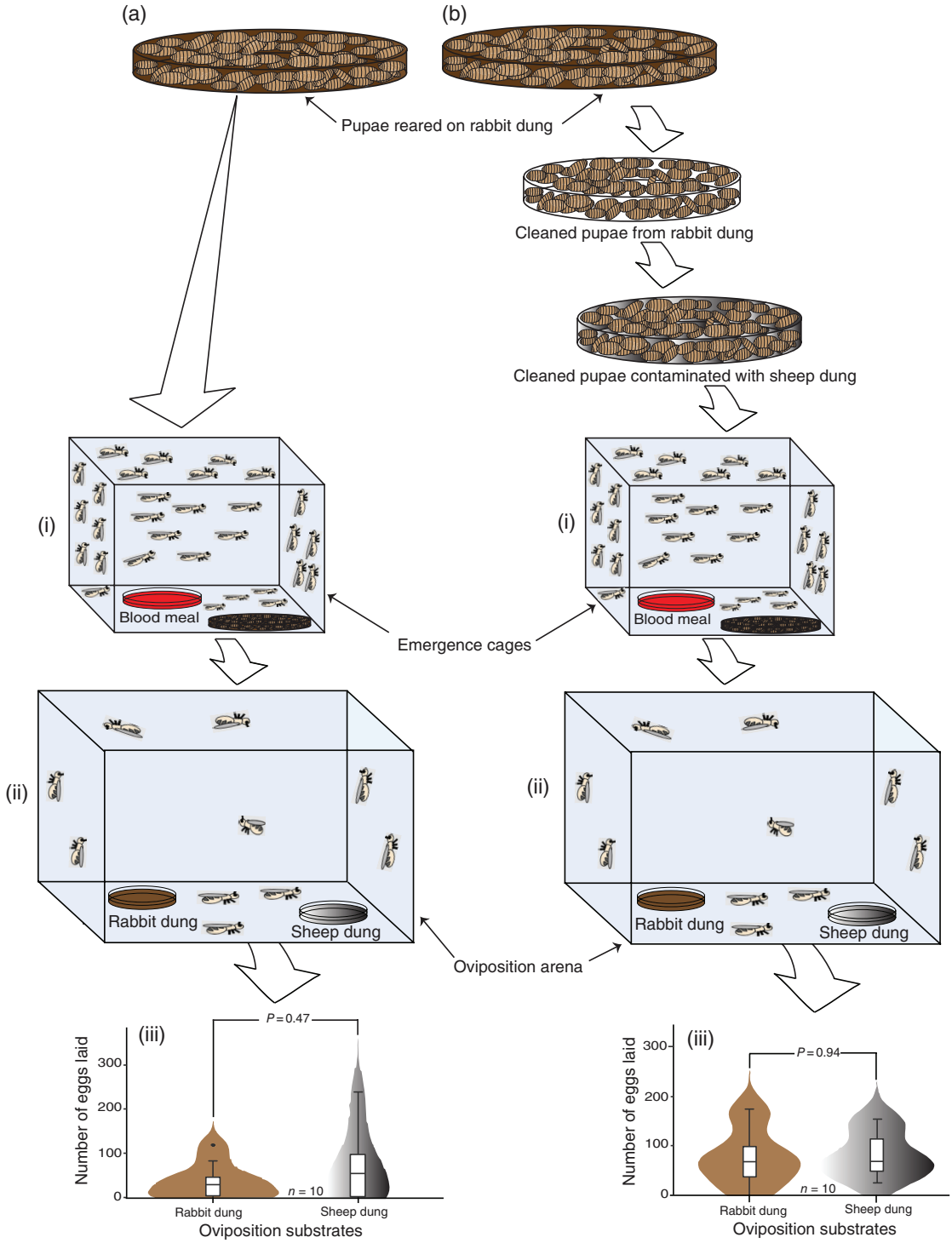

Fig. 5. Oviposition decision in gravid females of *S. calcitrans* is not affected by the larval chemosensory environment. (a) Oviposition bioassay using S. calcitrans gravid females from non-contaminated pupae: (i) Emergence cage, (ii) oviposition arena, (iii) Violin plots showing no significant difference (Mann–Whitney test; $p > 0.05$) in number of eggs laid on rabbit and sheep dung by gravid females emerged from non-contaminated pupae. (b) Oviposition bioassay using *S. calcitrans* gravid females from pupae contaminated with sheep dung: (i) Emergence cage, (ii) oviposition arena, (iii) Violin plots showing no significant difference (Mann–Whitney test; $p > 0.05$) in number of eggs laid on rabbit and sheep dung by gravid females emerged from pupae contaminated with sheep dung. Bar into each box show the median and those at the extremity of the box show the 25–75th percentiles, which are extended by whiskers indicating 1.5× the interquartile range from the 25th–75th percentiles; the shape denotes the density estimate and extends to extreme values. [Colour figure can be viewed at wileyonlinelibrary.com].

median number of eggs laid on sheep dung and donkey dung by gravid females that emerged from cleaned pupae were not significantly different ($U = 49$, d.f = 1, $P = 0.97$; Fig. 4c,i). Also, gravid females from uncleaned pupae laid the same median number of eggs on sheep dung and donkey dung ($U = 45.5$, d.f. = 1, $P = 0.762$; Fig. 4c,ii). In the bioassays with females reared on camel dung, gravid females from clean pupae laid more eggs on sheep dung than camel dung ($U = 15.5$, d.f = 1, $P = 0.01$; Fig. 4d,i). Similarly, gravid females from unclean pupae laid more eggs on sheep dung than camel dung ($U = 9$, d.f. = 1, $P = 0.002$; Fig. 4d,ii).

Chemical legacy hypothesis test. The chemical legacy hypothesis proposes that chemical cues coating pupae can influence adult oviposition substrate selection. Results of our two oviposition bioassays indicated that gravid females from non-contaminated and contaminated pupae did not exhibit different oviposition behaviour. Gravid females emerged from uncontaminated pupae ($U = 40$, d.f. = 1, $P = 0.47$; Fig. 5a,iii) and pupae contaminated with sheep dung ($U = 48.5$, d.f. = 1, $P = 0.94$; Fig. 5 a,iii) laid the same number of eggs on rabbit and sheep dung.

Discussion

We demonstrated that *S. calcitrans* larval preference for rearing substrate was not transferred to the adult stage during metamorphosis. When a rearing substrate was offered singly or paired with another substrate, L3 larvae of *S. calcitrans* preferred the substrate in which they had developed. That preference was disrupted when larvae were exposed to a substrate for 5 h. The ability of larvae to distinguish their rearing substrate from other substrates has also been observed in other holometabolous insects. Durisko and Dukas (2013) found that *D. melanogaster* larvae reared in a group are attracted by substrates that are occupied or that have been used by other larvae; the same larvae exhibit a significant aversion to fresh food. Similarly, larvae of *Ascia monuste orseis* reared on kale or cabbage prefer the same plants during their fourth and fifth instars when offered a choice including different plants (Santana & Zucoloto, 2011). In general, insects staying on a substrate for a period of time develop a preference for that substrate due to the adaptation of their olfactory sensory neurons (Iyengar *et al.* 2010). The aptitude to recognise their rearing substrate may prevent larvae of *S. calcitrans* from the deleterious effect of leaving a substrate. This was found in *Schistocerca americana*, where leaving one food source in search of another decreases food consumption, increases development time, suppresses growth rate, reduces larval weight, and elevates mortality (Bernays & Bright, 2001).

We found that *S. calcitrans* larvae transferred to a new substrate for 5 and 24 h tend to become adapted to this substrate and lose their preference for the original rearing substrate, even after having been returned to it. Neuser *et al.* (2005) explained that experience duration in insects varies from minutes to months, according to the species, age, and gender of the test organism, the strength of the rewarding or aversive stimulus, number of training repetitions, and type of bioassay. Dekker *et al.* (2006), Linz *et al.* (2013), and Crowley-Gall *et al.* (2016) all suggest that host shifts in insects presumably alter their olfactory system and consequently modify their behaviour.

Larval experience did not affect adult oviposition preference in *S. calcitrans*. Gravid females of *S. calcitrans* developed on camel and sheep dung, and emerged from clean pupae did not prefer to oviposit on their rearing substrate as hypothesised by the HHSP. This was also observed in gravid females that emerged from unwashed pupae, which contradicts the chemical legacy hypothesis. Consequently, our study found no evidence in support of the HHSP. Other studies have also failed to find an effect of larval experience on adult oviposition site preference. Rojas and Wyatt (1999) demonstrated that experience of the cabbage moth caterpillar *Mamestra brassicae* (Lepidoptera: Noctuidae) did not affect oviposition behaviour of their females. Oviposition preference in the milfoil weevil, *Euhrychiopsis lecontei* (Coleoptera: Curculionidae), is not an attribute fixed by larval experience (Solarz & Newman, 2001). Kerpel and Moreira (2005) did not find Hopkins' effect in *Heliconius erato* (Lepidoptera: Nymphalidae). In *Polygonia c-album* (Lepidoptera: Nymphalidae), larval diet also had no effect on egg-laying decisions of the adult female (Janz *et al.*, 2008). Our results imply that preference for particular dung types by female *S. calcitrans* for oviposition (Baleba *et al.*, 2019) is an innate rather than learned behaviour.

A possible explanation for a lack of connection between the larval rearing substrate and adult female oviposition preference is offered by the neo-Hopkins principle developed by Jaenike (1983), where host preference is determined by experience gained by the adult after emergence. The flight capability of adult *S. calcitrans* is relatively high (5 km; see Taylor *et al.*, 2010). During the flying period, adult *S. calcitrans* experience different kinds of visual and chemical cues that may shape adult behavioural choice. Barron (2001) also implicates the influence of genetic plasticity in insect host preference. Alternatively, *S. calcitrans* larvae feed on dung while their adults consume blood, which could also explain the absence of information transmission from larvae to adults. Furthermore, larvae of *S. calcitrans* are polyphagous; they feed and develop on a wide range of substrates, including dung (from camel, cow, horse, donkey and sheep), and decaying plant materials (such as hay, alfalfa, silage, sugarcane and compost). This polyphagous status is unfavourable for the HHSP. According to Janz et *al*. (2008), larval experience can only influence adult behaviour when the following conditions are fulfilled: low variation in substrate quality, predictable and recurring temporal heterogeneity, high spatial heterogeneity, and intermediate gene flow between spatial patches. So, it is not surprising that we did not find for the HHSP in *S. calcitrans*. Also, compared with specialist insects, the olfactory system of generalist insects is less sensitive (Zhang *et al.*, 2013; Conchou *et al.*, 2017), and according to the 'neural constraints hypothesis', generalist insects make less accurate decisions when selecting substrates (Sadtler *et al.*, 2014; Wang, 2017). This may render their behaviour difficult to predict. It is also important to note that where a positive influence of larval experience has been found on adult behaviour, Petit *et al.* (2017) proposed a role for insect phylogeny. *Stomoxys calcitrans* is one of 18 species in the genus *Stomoxys* (Zumpt, 1973), all

of which develop in animal dung (Hafez & Gamal-Eddin, 1959; Jeanbourquin & Guerin 2007; Cançado *et al.*, 2013). The wider subfamily Muscinae also rely on decaying plant matter, including dung, to complete their development, with few specific associations between Muscidae and other organisms (Haseyama *et al.*, 2015). Due to this genetic heritage, *S. calcitrans* may not have the capacity to learn oviposition preferences from an earlier life stage.

Overall, the current study has demonstrated that *S. calcitrans* larval preference is not transferred to the adult during metamorphosis. *Stomoxys calcitrans* gravid females did not prefer to lay eggs on the substrate in which they developed. Consequently, our findings do not match the expectations of the Hopkins's host selection hypothesis. This implies that preference by females for particular dung as an oviposition substrate is innate rather than learned. However, we suggest that there is a need for additional research on the applicability of the HHSP in generalist insects characterised by a high capacity for adaptation and flexibility in the use of substrates for development, as well assessment of different populations. Also, to better understand the lack of transmission of information from larvae to adult *S. calcitrans*, we recommend study of the development of the *S. calcitrans* mushroom body (structure in the insect brain essential for olfactory learning and memory). This will help to elucidate the changes undergone by the neurons forming this structure during *S. calcitrans* development.

Acknowledgements

This work was supported by the IBCARP camel, grant no. DCI-FOOD/2014/ 346-739 – mainly by European Union and Max Planck Institute for chemical ecology – *icipe* partner group to MNG. We also gratefully acknowledge the financial support for this research by the following organisations and agencies: Swedish International Development Cooperation Agency (Sida); U.K. Department for International Development (DFID); the Swiss Agency for Development and Cooperation (SDC); and the Kenyan Government. The views expressed herein do not necessarily reflect the official opinion of the donors. We thank Deutscher Akademischer Austauschdienst (DAAD), who provided SBSB with a doctoral scholarship through the ARPPIS-DAAD scholarship programme. We thank Heike Lutermann and all anonymous referees for their insightful comments on the manuscript.

The authors certify that they have no affiliations with or involvement in any organisation or entity with any financial interest, or non-financial interest in the subject matter or materials discussed in this manuscript.

Author contributions

SBSB conceived the research idea, designed the experiment, collected and analysed the data, and wrote the manuscript; CWW supervised the study, designed the experiment, and proofread the manuscript; DM supervised the study and proofread the manuscript; BT supervised and proofread the manuscript; MNG supervised the study, designed the experiment, and proofread the manuscript.

References

Baglan, H., Lazzari, C. & Guerrieri, F. (2017) Learning in mosquito larvae (*Aedes aegypti*): Habituation to a visual danger signal. *Journal of Insect Physiology*, **98**, 160–166.

Baleba, S.B.S., Torto, B., Masiga, D., Weldon, C.W. & Getahun, M.N. (2019) Egg-laying decisions based on olfactory cues enhance offspring fitness in *Stomoxys calcitrans* L. (Diptera: Muscidae). *Scientific Reports*, **9**, 3850.

Baldacchino, F., Muenworn, V., Desquesnes, M., Desoli, F., Charoenviriyaphap, T. & Duvallet, G. (2013) Transmission of pathogens by *Stomoxys* flies (Diptera, Muscidae): a review. *Parasite*, **20**, 26.

Barron, A.B. (2001) The Life and Death of Hopkins' Host-Selection Principle. *Journal of Insect Behavior*, **14**, 725–737.

Bernays, E. & Bright, K. (2001) Food choice causes interrupted feeding in the generalist grasshopper *Schistocerca americana*: further evidence for inefficient decision-making. *Journal of Insect Physiology*, **47**, 63–71.

Blackiston, D.J., Silva Casey, E. & Weiss, M.R. (2008) Retention of memory through metamorphosis: can a moth remember what it learned as a caterpillar? *PLoS One*, **3**, e1736.

Cançado, P.H., Ferreira, T., Piranda, E.M. & Soares, C.O. (2013) Sugarcane stems as larval habitat for the stable fly (*Stomoxys calcitrans*) in sugarcane plantations. *Pesquisa Veterinária Brasileira*, **33**, 741–744.

Carn, V.M. (1996) The role of dipterous insects in the mechanical transmission of animal viruses. *British Veterinary Journal*, **152**, 377–393.

Conchou, L., Anderson, P. & Birgersson, G. (2017) Host plant species differentiation in a polyphagous Moth: olfaction is enough. *Journal of Chemical Ecology*, **43**, 794–805.

Corbet, S.A. (1985) Insect chemosensory responses: a chemical legacy hypothesis. *Ecological Entomology*, **10**, 143–153.

Crowley-Gall, A., Date, P., Han, C., Rhodes, N., Andolfatto, P., Layne, J.E. *et al.* (2016) Population differences in olfaction accompany host shift in *Drosophila mojavensis*. *Proceedings of the Royal Society B: Biological Sciences*, **283**, 20161562.

Dalgaard, P. (2008) *Introductory Statistics with R. Statistics and Computing*. Springer New York, New York, NY.

Dekker, T., Ibba, I., Siju, K.P., Stensmyr, M.C. & Hansson, B.S. (2006) Olfactory shifts parallel superspecialism for toxic fruit in *Drosophila melanogaster* Sibling, *D. sechellia*. *Current Biology*, **16**, 101–109.

Durisko, Z. & Dukas, R. (2013) Attraction to and learning from social cues in fruit fly larvae. *Proceedings of the Royal Society B: Biological Sciences*, **280**, 20131398–20131398.

Ferrari, M.C.O., Messier, F. & Chivers, D.P. (2008) Threat-sensitive learning of predators by larval mosquitoes *Culex restuans*. *Behavioral Ecology and Sociobiology*, **62**, 1079–1083.

Friesen, K., Berkebile, D.R., Zhu, J.J. & Taylor, D.B. (2017) Augmenting laboratory rearing of stable fly (Diptera: Muscidae) larvae with ammoniacal salts. *Journal of Insect Science*, **17**, 21.

Gandolfi, M., Mattiacci, L. & Dorn, S. (2003) Preimaginal learning determines adult response to chemical stimuli in a parasitic wasp. *Proceedings of the Royal Society B: Biological Sciences*, **270**, 2623–2629.

Hafez, M. & Gamal-Eddin, F.M. (1959) Ecological studies on *Stomoxys calcitrans* L. and *Sitiens* Rond, in Egypt, with suggestions on their control. *Bulletin de la Société Entomologique d' Egypte*, **XLIII**, 245–283.

Haseyama, K.L.F., Wiegmann, B.M., Almeida, E.A.B. & Carvalho, C.J.B.d. (2015) Say goodbye to tribes in the new house fly classification: A new molecular phylogenetic analysis and an updated biogeographical narrative for the Muscidae (Diptera). *Molecular Phylogenetics and Evolution*, **89**, 1–12.

Hopkins, A.D. (1916). Economic Investigations of the Scolytid Bark and Timber Beetles of North America. U.S. Department of Agriculture Program of Work for 1917, p. 353.

Hu, P., Li, H., Zhang, H., Luo, Q., Guo, X., Wang, G. *et al.* (2018) Experience-based mediation of feeding and oviposition behaviors in the cotton bollworm: *Helicoverpa armigera* (Lepidoptera: Noctuidae). *PLoS One*, **13**, e0190401.

Iyengar, A., Chakraborty, T.S., Goswami, S.P., Wu, C.-F. & Siddiqi, O. (2010) Post-eclosion odor experience modifies olfactory receptor neuron coding in *Drosophila*. *Proceedings of the National Academy of Sciences*, **107**, 9855–9860.

Jaenike, J. (1978) On optimal oviposition behavior in phytophagous insects. *Theoretical Population Biology*, **14**, 350–356.

Jaenike, J. (1983) Induction of host preference in *Drosophila melanogaster*. *Oecologia*, **58**, 320–325.

Janz, N., SöDerlind, L. & Nylin, S. (2008) No effect of larval experience on adult host preferences in *Polygonia c-album* (Lepidoptera: Nymphalidae): on the persistence of Hopkins' host selection principle. *Ecological Entomology*, **34**, 50–57.

Jeanbourquin, P. & Guerin, P.M. (2007) Chemostimuli implicated in selection of oviposition substrates by the stable fly *Stomoxys calcitrans*. *Medical and Veterinary Entomology*, **21**, 209–216.

Kerpel, S.M. & Moreira, G.R.P. (2005) Absence of learning and local specialization on host plant selection by *Heliconius erato*. *Journal of Insect Behavior*, **18**, 433–452.

Lecomte, C. & Thibout, E. (1993) Pre- and post-imaginal experience in a specialist parasitoid *Diadromus pulchellus* (Hymenoptera.: Ichneumonidae). *Entomophaga*, **38**, 175–184.

Lehane, M.J. (2005) *The Biology of Blood-Sucking in Insects*. Cambridge University Press, New York, New York.

Leimar, O., Hammerstein, P. & Van Dooren, T.J. (2006) A new perspective on developmental plasticity and the principles of adaptive morph determination. *American Naturalist*, **167**, 367–376.

Linz, J., Baschwitz, A., Strutz, A., Dweck, H.K.M., Sachse, S., Hansson, B.S. *et al.* (2013) Host plant-driven sensory specialization in *Drosophila erecta*. *Proceedings of the Royal Society B: Biological Sciences*, **280**, 20130626.

Liu, S.-S. & Liu, T.-X. (2006) Preimaginal conditioning does not affect oviposition preference in the diamondback moth. *Ecological Entomology*, **31**, 307–315.

Margulies, C., Tully, T. & Dubnau, J. (2005) Deconstructing memory in *Drosophila*. *Current Biology*, **15**, R700–R713.

McDonald, J.H. (2009) *Handbook of Biological Statistics*. Sparky House Publishing, Baltimore, MD.

McCall, P.J. & Eaton, G. (2001) Olfactory memory in the mosquito *Culex quinquefasciatus*. *Medical and Veterinary Entomology*, **15**, 197–203.

Menda, G., Uhr, J.H., Wyttenbach, R.A., Vermeylen, F.M., Smith, D.M., Harrington, L.C. *et al.* (2013) Associative learning in the dengue vector mosquito, *Aedes aegypti*: avoidance of a previously attractive odor or surface color that is paired with an aversive stimulus. *The Journal of Experimental Biology*, **216**, 218–223.

Neuser, K., Husse, J., Stock, P. & Gerber, B. (2005) Appetitive olfactory learning in *Drosophila* larvae: effects of repetition, reward strength, age, gender, assay type and memory span. *Animal Behaviour*, **69**, 891–898.

Ning, S.Y., Yang, H.Y., Fan, D.S. & Feng, J.N. (2018) Influence of larval experience on preference of a subterranean insect *Delia antiqua* on *Allium* hosts. *Journal of Applied Entomology*, **142**, 263–271.

Olsson, P.-O.C., Anderbrant, O. & Löfstedt, C. (2006) Experience influences oviposition behaviour in two pyralid moths, *Ephestia cautella* and *Plodia interpunctella*. *Animal Behaviour*, **72**, 545–551.

Petit, C., Dupas, S., Thiéry, D., Capdevielle-Dulac, C., Le Ru, B., Harry, M. *et al.* (2017) Do the mechanisms modulating host preference in holometabolous phytophagous insects depend on their host plant specialization? A quantitative literature analysis. *Journal of Pest Science*, **90**, 797–805.

Pitzer, J.B., Kaufman, P.E., Tenbroeck, S.H. & Maruniak, J.E. (2011) Host blood meal identification by multiplex polymerase chain reaction for dispersal evidence of stable flies (Diptera: Muscidae) between livestock facilities. *Journal of Medical Entomology*, **48**, 53–60.

Proffit, M., Khallaf, M.A., Carrasco, D., Larsson, M.C. & Anderson, P. (2015) 'Do you remember the first time?' Host plant preference in a moth is modulated by experiences during larval feeding and adult mating. *Ecology Letters*, **18**, 365–374.

R Core Team (2018). R: A Language and Environment for Statistical Computing R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Ray, S. (1999) Survival of olfactory memory through metamorphosis in the fly *Musca domestica*. *Neuroscience Letters*, **259**, 37–40.

Reh-Hamburg (1924) Hopkins host-selection principle as related to certain Cerambycid beetles. *Zeitschrift für Induktive Abstammungs- und Vererbungslehre*, **32**, 89–91.

Rietdorf, K. & Steidle, J.L.M. (2002) Was Hopkins right? Influence of larval and early adult experience on the olfactory response in the granary weevil *Sitophilus granarius* (Coleoptera, Curculionidae). *Physiological Entomology*, **27**, 223–227.

Roberts, D. (2014) Mosquito larvae change their feeding behavior in response to kairomones from some predators. *Journal of Medical Entomology*, **51**, 368–374.

Rojas, J.C. & Wyatt, T.D. (1999) The role of pre- and post-imaginal experience in the host-finding and oviposition behaviour of the cabbage moth. *Physiological Entomology*, **24**, 83–89.

Sadtler, P.T., Quick, K.M., Golub, M.D., Chase, S.M., Ryu, S.I., Tyler-Kabara, E.C. *et al.* (2014) Neural constraints on learning. *Nature*, **512**, 423–426.

Santana, A.K. & Zucoloto, F. (2011) Influence of previous experience on the preference, food utilization and performance of *Ascia monuste* orseis Wild Larvae (Godart) (Lepidoptera: Pieridae) for three different hosts. *Neotropical Entomology*, **8**, 631–638.

Solarz, S.L. & Newman, R.M. (2001) Variation in hostplant preference and performance by the milfoil weevil, *Euhrychiopsis lecontei* Dietz, exposed to native and exotic watermilfoils. *Oecologia*, **126**, 66–75.

Taylor, D.B., Moon, R.D., Campbell, J.B., Berkebile, D.R., Scholl, P.J., Broce, A.B. *et al.* (2010) Dispersal of stable flies (Diptera: Muscidae) from larval development sites in a Nebraska landscape. *Environmental Entomology*, **39**, 1101–1110.

Taylor, D.B., Moon, R.D. & Mark, D.R. (2012) Economic impact of stable flies (Diptera: Muscidae) on dairy and beef cattle production. *Journal of Medical Entomology*, **49**, 198–209.

Thompson, J.N. (1988) Evolutionary ecology of the relationship between oviposition preference and performance of offspring in phytophagous insects. *Entomologia Experimentalis et Applicata*, **47**, 3–14.

Tully, T., Cambiazo, V. & Kruse, L. (1994) Memory through metamorphosis in normal and mutant Drosophila. *The Journal of Neuroscience*, **7**, 68–74.

Walker, A.R. (1990) Disease caused by arthropods. *Handbook on Animal Diseases in the Tropics*, 4th edn (ed. by M. M. H. Sewell and D. W. Brocklesby). Bailliere Tindall, London.

Wang, Y. (2017) Higher plasticity in feeding preference of a generalist than a specialist: experiments with two closely related *Helicoverpa* species. *Scientific Reports*, **12**, 1–12.

Zhang, H.-J., Faucher, C.P., Anderson, A., Berna, A.Z., Trowell, S., Chen, Q.-M. *et al.* (2013) Comparisons of contact chemoreception and food acceptance by larvae of polyphagous *Helicoverpa armigera* and oligophagous *Bombyx mori*. *Journal of Chemical Ecology*, **39**, 1070–1080.

Zumpt, F. (1973) The stomoxyine biting flies of the world: Diptera, Muscidae; taxonomy, biology, economic importance and control measures. *Gustav Fischer Verlag*. Stuttgart, Germany.

Accepted 17 April 2019
Associate Editor: Niklas Janz