Oviposition and host foraging cues of stable flies (*Stomoxys calcitrans*), and their potential as vectors of microbial host pathogens

by

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B.Sc. (Biological Sciences), Simon Fraser University, 2020

Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Pest Management

in the
Department of Biological Sciences
Faculty of Science

SIMON FRASER UNIVERSITY
Spring 2024

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Abstract

Stable flies, *Stomoxys calcitrans*, are blood-feeding ectoparasitic pests of cattle. I studied mechanisms that underlie oviposition and host foraging behaviour of stable flies and investigated whether they transmit pathogens. I show that odor and moisture of oviposition sites play distinguishable functional roles in the close-range attraction of gravid flies and their propensity to oviposit, and that ammonia – alone or with carbon dioxide – attracts flies and induces oviposition. I further show that several *Staphylococcus* microbes in the bovine skin microbiome attract host-foraging flies, and that ammonia and odorants emitted by these *Staphylococcus* microbes attract flies. Finally, I show that stable flies are attracted to the skin-dwelling, mastitis-causing bacterium *Staphylococcus aureus*, and that they transmit *S. aureus* from infected blood to sterile blood. My data infer the existence of a positive feedback loop. As *S. aureus* bacteria of afflicted cows proliferate, they attract even more flies which, in turn, worsen the infection.

Keywords: Stable fly; oviposition; cattle host-foraging; *Staphylococcus*; ammonia; pathogen transmission

11,160 flies in Chapter 2.

12,000 flies in Chapter 3.

3,648 flies in Chapter 4.

Tens (if not hundreds) of thousands more for preliminary trials, training, colony maintenance etc.

This thesis is dedicated to the cows whose blood was used to keep those darn flies alive.

More than 2,000 plates of bacteria.

More than 6,000 sterile “inoculating loops” (i.e., popsicle sticks…)

More bottles of ethanol than I care to admit.

This thesis is dedicated to my fellow germophobic microbiologists.

Four years of grad school.

About 25 or 30 mental breakdowns.

Oh, and a pandemic.

This thesis is dedicated to everyone who’s been struggling the last little while. It may not seem like it, but I promise it gets better.

Acknowledgements

Well, here we are. Almost exactly 4 years after starting my 2-year program (sorry it took me so long…) I've finally reached the end-

(Pending approval of this thesis)

Okay, I've almost reached the end. It goes without saying that no one does something like this without an immense amount of support, help, guidance, love and various other wonderful things from those around them, and I would be a truly awful human being if I didn't take a moment to thank the people who carried me through this process.

Firstly, my immeasurable gratitude to Regine and Gerhard Gries. You welcomed me into your lab as an undergrad in 2019 and made me feel at home right away. Thank you for your mentorship these past five years. Thank you for supporting me in my often-times unconventional way of making conference presentations. Thank you for your support and unwavering optimism through the many, many (many, many, many) times that I encountered roadblocks in the shape of negative data. Thank you for your endless generosity. Thank you for inspiring a love of science and research in all the students who have the privilege of being a part of the Gries lab. Thank you for everything. And, as always, thank you for the sweets.

Next, my thanks to Jaime Chalissery for taking me on as an undergraduate student. I had zero experience and had no idea what I was doing, so I owe you a lot for letting me join your team of undergrads and helping me get my foot in the door of research.

My sincerest thanks to Dr. Jane Fowler for agreeing to be on my committee. We asked you to join as a committee member right as you were starting up your lab at SFU, which I imagine was an incredibly busy time. And yet, you kindly agreed to be on my committee. Thank you so much for that, and everything. I cannot express enough how much I appreciate your helpful guidance and all the expertise you so generously lent me these past few years.

Thank you to Dr. Brandon Smythe for agreeing to be the external examiner for my defense. I am a huge fan of your research, and you were at the top of the list of people who I was hoping for, so I am extremely lucky and grateful to have you on board.

A very special thank you to all my amazing co-authors on Chapters 2-4. Adam Blake, for all your tireless help with statistics. Thank you for always being willing to sit down and go over absolutely everything I was confused about (which was absolutely everything). Sam Meraj, for being quite possibly the best molecular biologist on earth and for lending your immeasurable talents to not one but two of my research chapters. The work simply would not have happened without your support and expertise. Emerson Mohr, for stepping in and joining the team when we really needed another pair of hands, and for taking some of the most incredible photos of bacteria anyone has ever seen. Emma Kovacs, for being the best field partner anyone could ask for. Thank you for using so many of your own precious field season days helping me collect data. The car rides to and from the field sites were a highlight of my time as a grad student. Anand Devireddy, for your synthetic chemistry expertise. Much of Chapter 3 would not have been possible without the tireless work that you and Regine put into creating the (highly effective I might add) synthetic blends. Asim Renyard, for all the help with the statistics in Chapter 4. Figuring out how to analyze the colony data was no easy task, and it would not have been possible without your stats wizardry. Thank you for that. Thank you also for letting me distract you at work every five minutes with discussions about our favourite movies and pizza toppings.

To my friends who had to deal with my terrible work-life balance, but never ceased to check in with me and ask me to hang out (even though I almost always responded with, “oh sorry I can’t this time but next time for sure”). John, thank you for being the best friend ever and for making the trek up Burnaby Mountain IN THE SNOW just to hang out with me on the days I had too much on-campus work to do. Daniel, thank you for being the one to always check in to see how I was doing. Thank you for always taking the time to come up to campus to play basketball, or grab dinner, or just sit down and chat over a coffee. Jeff, thank you for the hangouts after work in the early days of my degree,

when things were at their most stressful. And thank you for not losing contact even after moving to that faraway place Toronto.

To Gena, thank you for making what should have been the hardest part of this degree the best part. Thank you for always getting me through the stressful times. Thank you for always being the most amazing person in the world (it's a little frustrating how great you are if I'm being completely honest). I love you sooooo much, you silly little guy.

Lastly, to my crazy family, thank you all. Dad, thank you for the rides home when I would be in the lab until 1:00am and the buses weren't running anymore. Haider, thank you for the constant support and for making time in your busy schedule to hang out with your boring younger brother. Ali, thank you for always checking in by surprising me at work at the times when I always seemed to need it the most. And most of all, thank you Mom for being there for me the past four years (and maybe also a couple before that as well). You were the one who gave me the kick in the butt I needed to do the Masters in the first place, and I can't thank you enough for that. Look Mom, I made it. I love you all so very much.

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Chapter 1: Introduction

1.1) Impact of stable flies

Stable flies, *Stomoxys calcitrans* (Diptera: Muscidae), are among the most problematic and prevalent biting fly pests worldwide. Numerous studies have been undertaken to determine the impacts of stable fly feeding on cows (e.g., physiological effects, blood loss, energy costs of defensive behaviours) and subsequent impacts on humans (e.g., economic and productivity losses) (Drummond et al. 1988).

1.1.1) The impacts of stable flies on cows

Stable flies have immense adverse impact on host cattle. This impact extends far beyond the pain and irritation caused by the flies' biting activity. Stable fly abundance and milk production by cows are negatively correlated (Bruce & Decker 1958). Cows treated to reduce fly loads gained weight significantly faster than untreated control cows who were left vulnerable to fly feeding (Cutkomp & Harvey 1958). Fly-pestered cows in dairy herds exhibit signs of restlessness, disturbed grazing, and increased bunching (huddling of cows to avoid stable fly attacks), often leading to antagonism between animals (Todd 1964). Defensive behaviours by cows, including tail flicks, leg stamps, and attempts to dislodge flies with their tongues, were also frequently observed (Todd 1964). Cows heavily engaged in defensive behaviours against flies were unable to rest or lie down. Importantly, a fly load of only 15 flies was sufficient to induce restlessness in cows (Todd 1964). Defensive behaviours increased with increasing numbers of flies per cow (Dougherty et al. 1993a; Mullens et al. 2006; Vitela et al. 2007). Induced defenses included head movements (8/min), front-leg lifts (8/min), back-leg lifts (3/min), ear movements (12/min), tail movements (69/min), and skin twitches (25/min) (Dougherty et al. 1993b). Stable fly attacks also caused severe interruptions to the cows' feeding activity (Doughtery et al. 1993a,b; 1994; 1995). In a 100-day study, loads of 50 and 100 flies per calf lowered weight gain by 0.9 kg/day and 0.22 kg/day, respectively, reducing feed efficiency by 12.9% and 10.9%, respectively (Campbell et al. 1977). Stable fly population estimates at several feedlots and one dairy farm were linked to weight losses

of 2.3 and 3.51 kg/animal during the summers of 1980 and 1981, respectively (Berry et al. 1983). Cows wearing insecticide-treated ear tags exhibited less leg stamping and kicking than untreated cows but neither grazing nor milk yield differed between treated and untreated cows (Harris et al. 1987). A 10.6% lower mean daily weight gain was reported for 14- to 15-month-old cows exposed to stable fly attacks (Catangui et al. 1993). In a similar study, the mean daily weight gain of stable fly-exposed, 12- to 14-month-old cows was 9% lower (Catangui et al. 1995). Finally, a 0.2-kg/day lower weight gain in cows was attributed to stable fly feeding activity (Campbell et al. 2001). Importantly, when the stable fly stress was removed after the 12-week trials were terminated, and the animals were fed in a feedlot, there was no compensatory weight gain by cattle (Campbell et al. 2001).

Almost 72% of lower weight gain due to cow bunching was attributed to heat stress (Wieman et al. 1992), with the other 28% attributed to more direct effects including fly biting and energy allocated to defensive behaviours. Interestingly, neither a 3 ºC increase in body temperature nor an incremental increase of 6 flies per leg lowered the cattle's weight gain, but both factors in combination did (Campbell et al. 1993).

Blood loss from stable fly feeding activity significantly affects host animals. Each fly was estimated to consume between 9.3–11.63 mg of blood per day (Dougherty et al. 1994, 1995). With hundreds of flies feeding daily on a cow, blood losses are staggering.

Estimates of reduced weight gain by cattle due to stable fly feeding vary widely [e.g., 90 kg over 100 days (Campbell et al. 1977); 3.51 kg/animal over an entire summer (Berry et al. 1983)] but the flies' significant downstream effects on the health of host animals are undisputed.

1.1.2) Impacts of stable flies on humans

As humans are not the primary hosts of stable flies, fly feeding is not the main adverse effect on them. Rather, the flies' indirect effect on humans lies in the lower productivity they cause in cattle industries.

In 1954, the United States Department of Agriculture (USDA) estimated annual losses due to stable flies at ~$20 million (USDA 1954). By 1965, estimates had increased to $142 million (USDA 1965). With an estimated loss of $16.40 per head of cattle (Drummond et al. 1981), and with 12.9 million cattle in America at the time, the total loss amounted to $211.56 million per year. When also factoring in an estimated 5% lower milk production due to stable fly feeding, the total monetary losses caused by stable flies were estimated at 398.9 million USD per year in 1981 (Drummond et al. 1981). Ten years later, total annual losses due to stable flies in both feedlots and dairies were projected to be $432 million (Kunz et al. 1991). By 2001, the per-animal loss was estimated at $33.26, more than twice that 20 years earlier (Campbell et al. 2001). Finally, stable fly bites also damage cow skin, resulting in leather products of poorer quality (Stosic et al. 2000).

The economic threshold for stable fly control – the point at which it costs more money to not undertake control measures – has been estimated to be fewer than two flies per cow leg (Campbell et al. 1987). This means that the presence of two or more flies on a single leg of a cow should trigger fly control measures to avoid downstream economic losses. This seemingly conservative estimate highlights the extent of economic impact that stable flies exert, especially considering that fly populations are rarely ever that low.

1.2) Stable fly life cycle

Both male and female flies need to ingest blood to become sexually mature, and females require a blood meal to produce eggs (Foil and Hogsette 1994). Females continue egg-laying until they die. A clutch of eggs may contain between 60 and 130 1-mm long white eggs. The stable fly life cycle has four stages: egg, larva (three instars), pupa and adult. First-instar larvae (< 2 mm in length and nearly translucent) hatch 12–24 h after egg laying. Within the next 2–3 days, they grow and molt into whitish 2nd instars (7–8 mm) which become reddish 3rd instars (>10 mm) 3–4 days later. Finally, the outer layer of 3rd instars hardens to form a puparium (5 mm), inside which the larva becomes a pupa. The pupal stage takes 4–10 days, or longer if environmental conditions are suboptimal for adult eclosion. Finally, the fully developed adult fly ecloses from the puparium. Almost

immediately after eclosion, adult flies are able to forage for cattle and to ingest blood. Within 3–5 days after eclosion, they are ready to mate, and 1–2 days later, females are able to produce eggs (Foil and Hogsette 1994). Female flies live up to 4.5 weeks under laboratory conditions but likely not as long in the wild (Killough and McKinstry 1965).

1.3) Stable flies and semiochemicals

An overarching theme of my thesis is the exploitation of attractive semiochemical gases and odorants by stable flies to locate resources such as host animals and oviposition sites. In this section, I review some of those semiochemicals that have been investigated as potential attractants for stable flies. A comprehensive list of investigated semiochemicals is presented in Table 1.1.

1.3.1) CO_2

As many as 19 studies have investigated the effect of carbon dioxide (CO_2) on stable fly activity. This large body of literature prompted me to dedicate an entire subsection of my introductory chapter to the role of CO_2.

Pre-1990s studies on CO_2

In early field trials (Hoy 1970), Malaise traps releasing 3 L of CO_2/min captured 3-times more flies than traps baited with carbon monoxide (CO) or left unbaited. Investigating how CO_2 and human skin odor affect stable fly activity in a wind tunnel, Gatehouse & Lewis (1973) concluded that both stimuli enhanced flight activity, with CO_2 activating flies and odor guiding flies towards resources. Vale (1980) found that CO_2 attracted *Stomoxys* spp. (not specifically *S. calcitrans*) in field trials. Similarly, CO_2-baited Alsynite traps captured 8,000–10,000 flies per week in field settings (Gersabeck et al. 1982). CO_2 increased the flight activity of stable flies, and CO_2 in combination with human breath odorants synergistically affected the flies' behavioral responses (Warnes & Finlayson 1985a). These findings, along with those of Gatehouse & Lewis (1973), supported the concept that CO_2 is only one component of the host cue complex. In a follow-up study, CO_2 induced anemotaxis of flies (oriented movement in response to a

current of airflow), as did human breath (Warnes & Finlayson 1985b). Finally, electroantennogram (EAG) responses to CO_2 increased logarithmically with increasing CO_2 concentration, plateauing at around 2% CO_2 (Warnes & Finlayson 1986).

1990–1999

At Nairobi National Park in Kenya, traps baited with CO_2 alone or in combination with 1-octen-3-ol captured significantly more *Stomoxys* spp. than traps baited with 1-octen-3-ol alone (Mihok et al. 1996). As unbaited control traps were not part of the experimental design, the effect of only 1-octen-3-ol as a trap bait could not be ascertained. In follow-up experiments, traps baited with CO_2 alone and in combination with 1-octen-3-ol at several concentrations captured significantly more flies than unbaited control traps (Mihok et al. 1996). Similarly, Alsynite traps baited with both CO_2 (released from dry ice) and 1-octen-3-ol captured significantly more flies than traps baited with either CO_2 or 1-octen-3-ol, or left unbaited (Cilek 1999). However, these results could not be fully replicated the following year. In field trials near Lake Victoria (Kenya), biconical traps releasing CO_2 at 5 L/min captured more stomoxyine flies than unbaited control traps (Ahmed & Mihok 1999). In a dense forest, a release rate of only 2.5 L of CO_2/min was sufficient to obtain equivalent results (Ahmed & Mihok 1999). Altogether, the data demonstrate that CO_2 as a semiochemical gas for stable flies is effective in diverse geographic locations.

In wind tunnel bioassays, CO_2 induced upwind flight in flies (Schofield & Brady 1997), with greater proportional flight activity occurring when CO_2 was being released at concentrations of 0.12%, 0.06%, 0.012%, and 0.006% above ambient (Schofield et al. 1997). The same CO_2 concentrations elicited sensory responses in electroantennogram (EAG) recordings (Warnes & Finlayson 1985b). With and without elevated CO_2 levels, the resting times of flies (time spent not flying) were 7.1 s and 16.6 s, respectively (Schofield et al. 1997), further indicating that CO_2 induced flight activity. Finally, more landings occurred on black objects (i.e., “surrogate hosts”) in wind tunnel bioassays when CO_2 was being released, suggesting again that CO_2 prompted flies to forage for hosts. Whereas CO_2 activates host foraging in flies, the question remained as to which semiochemicals guide flies toward their hosts.

2000 – Present

With increasing frequency of exhaling human breath (comprising CO_2 and breath odorants), the breath's attractiveness to stable flies increased (Alzogaray & Carlson 2000). CO_2 on its own induced activation, orientation and probing responses in flies (Alzogaray & Carlson 2000), once again indicating the importance of CO_2 as an activator of host foraging behavior. Ox and calf odors were most and least attractive to flies, respectively (Torr et al. 2006). These findings were attributed to quantitative and/or qualitative differences in odor composition between hosts, supported by the fact that adding CO_2 to calf odor made it significantly more attractive, and as attractive as ox odor.

CO_2 is also an important oviposition cue for gravid female stable flies. That gravid females laid significantly more eggs on horse feces than on cow feces (Jeanbourquin & Guerin 2007a) was attributed to differential CO_2 emission from these fecal sources. While at the onset of these 24-h bioassays both fecal sources released CO_2 at similar levels (400–410 ppm), 8 h later CO_2 emission from horse feces had risen to 430–440 ppm and from cow feces had declined to ~390 ppm. Female flies were significantly more attracted to CO_2 (emanating from dry ice) than to controls in three out of four field sites (Phasuk et al. 2016), whereas equivalent responses of males were observed in only one out of four field sites. These data imply sex-specific differences in the flies' attraction to CO_2, or that female flies use CO_2 to locate both vertebrate hosts and potential oviposition sites.

Vavoua, Malaise or Nzi traps baited with a mixture of fermented cow urine and goat feces, or a mixture of fermented cow urine and fermented straw, captured significantly more *Stomoxys* spp. than unbaited control traps (Tunnakundacha et al. 2017), with the baits' attractiveness credited to CO_2 release from fermenting materials. Similarly, Knight Stick sticky traps baited with CO_2 captured significantly more flies than unbaited control traps (Hogsette & Kline 2017). Altogether, these results stress the importance of supplementing 'vison-based' traps with olfactory cues for optimal attractiveness to flies.

1.3.2) Electroantennograms (EAGs) and gas chromatographic-electroantennographic detection (GC-EAD) analyses

In EAG recordings, fly antennae responded to human breath, odors originating from the nose of cows, and cattle odors filtered to remove CO_2 (Warnes & Finlayson 1986). That antennae also sensed odors from fresh cow feces is hardly surprising considering that stable flies use cow feces as an oviposition resource (see section 1.4.2 below). Interestingly, acetic acid induced negative deflections in EAG recordings. EAG-active chemicals included 3-methylphenol, octan-1-ol, 1-bromooctane, octan-3-ol, octanal, and 2-octanone (Schofield et al. 1995), all of which are components of host odor, implying that they may serve a role in host location or recognition by flies.

Birkett et al. (2004) ran EAGs with dipteran antennae, including those of stable flies, on many chemicals, of which only a few are being described here, with a more complete list reported in Table 1.2. Among the EAG-active chemicals that elicited antennal responses were *m*-cresol, *p*-cresol, 4-methyl-2-nitophenol, linalool and citronellol (Table 1.2). Interestingly, only the *meta*- and *para*-configuration, but not the *ortho*-configuration, of cresol was EAG-active. Inactive were propyl benzene, phenol, indole and skatole (Table 1.2). That indole and skatole did not elicit antennal responses was particularly surprising, considering that they are common odor constituents of the fecal matter that stable flies often use for oviposition.

Although the physical structure of sensory organs and their underlying sensory response mechanisms are not the focus of my thesis, it is nevertheless helpful, and interesting, to have a basic understanding of them. There are four major types of olfactory sensilla on stable fly antennae: basiconic, trichoid (short, medium, long), clavate and coeloconic (Tangtrakulwanich et al. 2011). In that same study by Tangtrakulwanich et al. (2011), both indole and phenol were EAG-active, contrasting with results obtained by Birkett et al. (2004). Also active was butyric acid, which elicited greater antennal responses by males than by females. Conversely, isovaleric acid elicited greater responses from female antennae than from male antennae, suggesting it may serve a role in oviposition site selection.

Butanoic acid, α-humulene, acetophenone, isovaleric acid, and borneol originating from horse feces, as well as heptan-1-ol originating from cow feces, all elicited strong

responses from stable fly antennae (Jeanbourquin & Guerin, 2007a), suggesting that these compounds might attract stable flies to animal feces for oviposition. EAG-active terpenes, including citronellene, D-limonene, β-caryophyllene, β-cyclocitral, and α-humulene (Jeanbourquin & Guerin, 2007a), may help guide flies to (fermenting) plant material in animal waste. Among the many chemicals screened for EAG activity (Table 1.2), 1-octen-3-ol, dimethyl trisulfide, and β-cyclocitral elicited the strongest antennal responses (Jeanbourquin & Guerin 2007b). Synthetic analogues of dimethyl trisulfide, *p*-cresol and several others (Table 1.2) also induced EAG responses, especially at higher doses. Obviously, many chemicals prompt electrophysiological responses by flies (Jeanbourquin & Guerin 2007a & b; Table 1.2), and fly attraction to oviposition sites is likely mediated by semiochemical cues.

Lastly, in EAG recordings and in GC-EAD analyses, several compounds emanating from vinasse, including butanoic acid, hexan-1-ol, pentanoic acid, and heptan-1-ol, elicited antennal responses (Table 1.2; Serra et al. 2017). However, electrophysiological responses differed based on the locations from which the vinasse originated.

1.3.3) Other semiochemicals tested

Odor sources such as the breath, skin or feces of host animals have been studied for their effect on stable fly attraction. These types of studies provide useful information as to which sources are attractive to flies. For example, Vavoua traps baited with urine from cow, buffalo, waterbuck or camel, or with feces from rhinoceros, elephant or hippopotamus, all captured more *Stomoxys* spp. flies than unbaited control traps (Mihok et al. 1995). Similarly, rumen digesta induced significantly more fly activation in wind tunnel bioassays than control odors (Jeanbourquin & Guerin, 2007b). Finally, human breath and live guinea pig odors induced anemotactic responses by flies in wind tunnel bioassays (Warnes & Finlayson 1985b). Even more intriguing, cattle sebum induced arrestment responses as well as continued searching behaviour in flies (Warnes & Finlayson 1985b). These results are particularly important because they reveal that skin odors can induce foraging responses in flies. If the skin odors originate from the cattle's

microbiome, the specific microbes causing the attraction should be identified. This is one of my research objectives of Chapter 3.

Animals are not the only source of semiochemical attractants for stable flies. Plant matter as well as industrial by-products are also attractive to flies, particularly in the context of oviposition. Large numbers of flies were captured on white, adhesive-coated Nzi, Vavoua, Model H, and Ngu traps at a pineapple plantation 2 days after harvest when break down of plant material starts (Solorzano et al. 2015). That even more flies were captured 15–28 days after the study onset implies that the flies responded to organic material which was increasingly breaking down and fermenting. Increased fermentation leads to increased release of CO_2 which – as discussed in 1.3.1 – is a potent activator and powerful attractant for flies. As a by-product of ethanol production during sugarcane fermentation, vinasse remains after ethanol and other products have been removed. Being produced in the process of fermentation, it is not surprising that vinasse attracts stable flies. Large numbers of flies were captured on cylindrical sticky traps in an area immediately after treating it with vinasse (Souza et al. 2021), likely due to a significant level of fermentation happening at that point in time. In Y-tube bioassays, vinasse alone, and in combination with 2- or 5-day fermented sugarcane straw, was more attractive to mated female flies than the control, suggesting that vinasse may also be an oviposition site attractant (Serra et al. 2017). In field studies, traps baited with vinasse captured significantly more flies than traps baited with filter cake, vinasse and filter cake, or left unbaited (Serra et al. 2017).

Phenols and cresols have also been extensively tested for stable fly attraction, but results varied (Holloway and Phelps 1991; Djiteye et al. 1998; Cilek 1999; Mihok et al. 2007; Jeanbourquin & Guerin 2007b; Tangtrakulwanich et al. 2015; Zhu et al. 2016; Lehmann et al. 2023). As trap baits, 4-methylphenol and 3-*n*-propylphenol released at 0.7 and 0.15 mg/h, respectively, had no effect on fly captures (Holloway & Phelps 1991). Chemical blends including *m*-cresol and 1-octen-3-ol increased stable fly captures in various field sites, but data were not analyzed statistically (Djiteye et al. 1998). A blend of 1-octen-3-ol, 3-*n*-propylphenol and 4-methylphenol released at 0.7 mg/h and 19.6 mg/h increased fly captures in Alsynite traps by 3-fold (Cilek 1999). Conversely, the

attractiveness of 1-octen-3-ol as a bait for Nzi traps could not be enhanced by adding a binary blend of 4-methylphenol and 3-*n*-propylphenol, 2-methoxyphenol, or 2-methoxy-4-methylphenol (Mihok et al. 2007). Furthermore, traps baited with both acetone and 4-methoxyphenol attracted as few flies as unbaited control traps (Mihok et al. 2007). In wind tunnel bioassays, *p*-cresol activated and attracted more flies than a control stimulus (Jeanbourquin & Guerin 2007b). In laboratory bioassays, 10-µL samples of low concentrations (1 µg/10 µL) of phenol, *m*- and *p*-cresol (which occur in cattle manure slurry), were more attractive to flies than the control (Tangtrakulwanich et al. 2015). At a medium dose (10 µg/10 µL), 10-µL samples of phenol remained attractive, whereas the two cresols became repellent; at a high dose (100 µg/10 µL), phenol and both cresols were as unattractive as the control, indicating dose-dependent attractiveness of these compounds (Tangtrakulwanich et al. 2015). In field experiments, Alsynite traps baited with a binary blend of phenol and *m*-cresol, or phenol and *p*-cresol, captured more flies than unbaited control traps (Tangtrakulwanich et al. 2015). Similarly, white panel sticky traps baited with phenol, *m*-cresol, or *p*-cresol, all captured more flies than unbaited control traps (Zhu et al. 2016). In laboratory bioassays, adhesive tape treated with *m*-cresol attracted and captured more flies than the untreated control tape but only after 24 h had lapsed after the treatment (Zhu et al. 2022). Equivalent effects were not observed when the tape was treated with phenol or *p*-cresol. In laboratory dose-response bioassays that tested *m*-cresol at 1, 3, 6 and 10%, *m*-cresol at 10% was most attractive to flies (Zhu et al., 2022). In a field experiment, however, *m*-cresol at 10% was not effective as a trap bait (Zhu et al., 2022), possibly because the dose was too low to overcome the background 'noise' in the field setting. In a further field experiment aimed to develop a push-pull strategy for stable fly control, *m*-cresol (1 mg dissolved in 100 mL of hexane) added to cylindrical sticky traps reduced fly numbers by 17% and 21% in each of two years (Lehmann et al. 2023).

Only a few acids have been investigated for their effect on stable fly behavior. In field trials, acetic acid was repellent to *Stomoxys* spp. (Vale 1980), and Vavoua traps baited with lactic acid did not capture more *Stomoxys* spp. than unbaited control traps (Mihok et al. 1995). In wind tunnel experiments, however, butanoic acid activated and attracted flies (Jeanbourquin & Guerin 2007b).

Although ammonia is prevalent in the environment, it has rarely been tested on stable flies. Ammonia released at 1.1 and 2.2 mg/L of air induced probing responses by stable flies, but a lower concentration of ammonia (0.56 mg/L of air) had no effect (Hopkins 1964). As these findings could not be reproduced in a follow-up study (Gatehouse 1970), more studies are needed to demonstrate whether, or not, ammonia modifies the behaviour of stable flies in the context of host foraging or oviposition. Thus, the effects of ammonia are investigated in Chapters 2 and 3 of my thesis.

Many other materials and chemicals have been tested for stable fly attraction. Conspecific fly feces collected continuously over 3 days was more attractive to flies than feces collected over 1 day or 6 days (Carlson et al. 2000), suggesting that microbial metabolites emanating from conspecific feces are involved in fly attraction, and that the most attractive microbial metabolites occur after 3 days. Conversely, Nzi traps baited with both acetone and stable fly feces were not more attractive than traps baited with acetone alone or left unbaited (Mihok et al. 2007). In wind tunnel bioassays, rumen digesta and dimethyl trisulfide, each activated flies more than a control stimulus did, whereas skatole neither activated nor attracted flies (Jeanbourquin & Guerin 2007b). In the context of oviposition, β-citronellene and carvone applied on wet sand prompted more egg laying by stable flies than camphene, (*R*)-limonene, *m*-cresol, and *p*-cymene (Baleba et al. 2019a). In follow-up field studies, traps baited with β-citronellene captured more gravid female flies than unbaited control traps (Baleba et al. 2019a). Conversely, traps baited with *m*-cresol, carvone, and binary and ternary blends of (*i*) β-citronellene and carvone, (*ii*) β-citronellene and valencene, and (*iii*) carvone, valencene and γ-terpinene, all did not capture more flies than unbaited control traps (Baleba et al. 2019a). Altogether, these results seem to indicate that β-citronellene serves an attractant in oviposition site selection.

1.4) Oviposition

1.4.1) General oviposition in dipterans

Oviposition sites and strategies of flies (Diptera) are diverse and guild-specific. Female spotted-winged *Drosophila*, *Drosophila suzukii* (Drosophilidae), lay their eggs in small fruits with varying degrees of ripeness (Lee et al. 2011). Gravid female blow flies (Calliphoridae) seek carrion of a specific age, temperature, moisture, and the presence or absence of conspecific larvae (Yang & Shiao 2012; Charabidze et al. 2015; Hans et al. 2019; Kotzé & Tomberlin 2020), whereas female house flies, *Musca domestica* (Muscidae), deposit their eggs on animal feces. To inoculate mammalian hosts with their eggs, human botflies, *Dermatobia hominis* (Oestridae), use mosquitoes and ticks as mechanical vectors that deliver the eggs when they pierce through the skin of mammalian hosts (Maier & Hönigsmann 2004). Craneflies (Tipulidae) lay their eggs in moist soils (Laughlin 1958) and parasitoid tachinid flies (Tachinidae) lay their eggs in the feeding path of herbivores to be ingested by prospective hosts (Grenier 1988).

Both olfactory cues (see 1.3 above) and visual cues mediate attraction of gravid female flies and affect their oviposition decisions. In the vinegar fly *Drosophila melanogaster* (Drosophilidae), acetic acid induces oviposition in gravid females but deters oviposition in unmated females (Joseph et al. 2009). While ethanol induces oviposition by *D. melanogaster* and *D. santomea*, it is aversive to other female flies (e.g., *D. mauritiana*) that lack ethanol tolerance (Sumethasorn & Turner 2016). Cedrol is an oviposition attractant to gravid female *Anopheles gambiae* mosquitoes (Culicidae) (Lindh et al. 2015), and a seven-component semiochemical blend emanating from rotten chicken liver induces oviposition by gravid female screwworms, *Cochliomyia macellaria* (Calliphoridae) (Zhu et al. 2013). Female onion flies, *Delia antiqua* (Anthomyiidae), lay more eggs when other flies are present on oviposition resources (Hoshizaki et al. 2020), suggesting that visual cues affect oviposition decisions. Similarly, traps baited with dimethyl trisulfide (DMTS) and covered with black paper captured more gravid female blow flies than DMTS-baited traps covered with yellow paper, indicating that a specific bimodal cue complex signifies suitable oviposition sites to gravid female flies (Brodie et al. 2014). Interestingly, microbes – deposited by female house flies together with their eggs – proliferate on the egg surface and alter subsequent oviposition decisions by conspecific flies on oviposition resources, inducing or deterring further egg-laying

depending on the time that has elapsed following the first oviposition bout (Lam et al. 2007).

1.4.2) Oviposition and larval development in stable flies

Stable fly larvae can develop in a wide variety of substrates (Machtinger et al. 2014), including silage and other fermenting or decomposing organic matter which may also serve as overwintering sites (Lysyk 1993; Berkebile et al. 1994; Taylor & Berkebile 2011; Cook et al. 2018). Expectedly then, gravid female stable flies seeking oviposition sites are less selective than some of their dipteran relatives. Female stable flies lay eggs in cow and horse feces – particularly aged horse feces (Albuquerque & Zurek 2014; Machtinger et al. 2014) – and preferentially oviposit on donkey and sheep feces in Africa (Baleba et al. 2019a), with β-citronellene and carvone attracting flies to these resources (see section 1.3.3 above). Stable flies lay eggs also in rotting vegetation (Solorzano et al. 2015; Serra et al. 2017), grass clippings (Ware 1966), silage mounds (Lysyk 1993), and spilled cattle feed (Meyer & Peterson 1983). Moreover, gravid females do not preferentially oviposit in the same type of medium in which they themselves have developed (Baleba et al. 2019b), further indicating plasticity in their choice of oviposition sites. With so many oviposition site options, it would be interesting to determine their common characteristics. This is my research objective in Chapter 2.

Although stable flies can lay eggs in a plethora of oviposition sites, oviposition decisions seem well informed. Gravid flies avoided oviposition substrate containing con- or hetero-specific larvae as well parasitic mites (Baleba et al. 2020). The flies' oviposition decisions were likely informed by semiochemicals because flies avoided such occupied oviposition sites also in complete darkness when the flies were reliant exclusively on olfactory cues. Egg-laying was also reduced on substrates treated with either catnip oil, or its major chemical constituents (nepetalactones) (Zhu et al. 2012). In a context other than oviposition, many potential stable fly repellents have been tested, including lemongrass oil (Baldacchino et al. 2013), catnip oil (Zhu et al. 2009), mixtures of essential oils (Woolley et al. 2018), certain fatty acids (Mullens et al. 2009), mixtures of insecticides (Fankhauser et al. 2015), and various plant-based compositions (Showler

2017). Unraveling the attractive characteristics of preferred oviposition sites is the overarching aim in Chapter 2 of my thesis.

The diversity of stable fly oviposition sites implies common characteristics such as moisture content and odor profile. Relative moisture content of oviposition sites was correlated with both the abundance of stable fly larvae (Friesen et al. 2016) and the number of adult flies emerging from oviposition sites (Wienhold & Taylor 2012). Similarly, during periods of increased rainfall more stable fly adults were present (Mullens & Peterson 2005). Odorants such as dimethyl trisulfide, butanoic acid, and *p*-cresol were found in headspace volatiles of both cow and horse feces, and were shown to elicit responses from stable fly antennae (Jeanbourquin & Guerin 2007a,b; see also section 1.3.2 above). Moreover, white panel sticky traps baited with the fecal volatiles phenol, *p*-cresol, or *m*-cresol, each captured more flies than unbaited control traps (Zhu et al. 2016). Although the reproductive status of these captured flies were not reported, the flies were likely gravid and in search for oviposition sites because phenol and cresols commonly emanate from fecal matter (Jeanbourquin & Guerin 2007a,b) and other decomposing organic matter (Serra et al. 2017) that stable flies seek as oviposition sites.

1.5) Microbe-mediated attraction to hosts

1.5.1) Insect-Microbe Interactions

Interactions between insects and microbes have been extensively studied (Davis et al. 2013). The aphid parasitoid wasp *Aphidius colemani* (Hymenoptera: Braconidae) is attracted to volatiles emitted from *Bacillus* spp. isolated from the potato aphid, *Macrosiphum euphorbiae* (Hemiptera: Aphididae), and the green peach aphid, *Myzus persicae* (Hemiptera: Aphididae) (Goelen et al. 2020), suggesting that microbes may play a role during host location by parasitoids. The parasitoid wasp *Trissolcus basalis* (Hymenoptera: Scelionidae) is attracted to various microbes isolated from nectar, including *Staphylococcus epidermidis, Terribacillus saccharophilus, Pantoea* sp.*,* and *Curtobacterium* sp. (Cusumano et al. 2022). These microbes and their volatile organic compounds (VOCs), including 2-methoxy-*p*-cymene, glutaric acid dimethyl ester, methyl

dihydrojasmonate, and 2,5-dimethylbenzaldehyde, were hypothesized to be the means by which parasitoids locate floral sources for nectar-feeding (Jervis et al. 1993; Cusumano et al. 2022). Moreover, nectar-foraging common house mosquitoes, *Culex pipiens* (Diptera: Culicidae), are attracted to metabolites of the yeast *Lachancea thermotolerans* which dwells in floral nectar of common tansies, *Tanacetum vulgare* (Peach et al. 2021). Similarly, the aphid parasitoid *Aphidius ervi* (Hymenoptera: Braconidae) is attracted to odors from several nectar-dwelling yeasts, including *Aureobasidium pullulans, Metschnikowia gruessii*, and *Metschnikowia reukaufii* (Sobhy et al. 2018). The pestiferous lesser grain borer, *Rhyzopertha dominica* (Coleoptera: Bostrichidae), is attracted to VOCs emitted from several wheat-colonizing fungi, suggesting a role of VOCs in the attraction of grain borer beetles to host plants (Van Winkle et al. 2022). Larvae of the cotton leafworm, *Spodoptera littoralis* (Lepidoptera: Noctuidae), are attracted to, and feed on, the yeasts *Metschnikowia hawaiiensis*, *M. lopburiensis* and *Cryptococcus nemorosus,* likely guided by yeast VOCs (Ljunggren et al. 2019). The Gram-positive bacterium *Staphylococcus xylosus*, isolated from aphid honeydew, attracts the black garden ant, *Lasius niger* (Hymenoptera: Formicidae) (Fischer et al. 2015), implying a role of microbes in sustaining the mutualistic relationship between aphids and ants. When strains of Brewer's yeast, *Saccharomyces cerevisiae*, were experimentally mutated to suspend the release of specific VOCs, they failed to attract *D. melanogaster* vinegar flies (Christiaens et al. 2014). Yet, when ethyl acetate – which was not released from the mutant yeast strain – was experimentally added to the mutant's volatile blend, its original attractiveness to vinegar flies was restored (Christiaens et al. 2014). Depending on the medium (corn syrup or sucrose) used for yeast growth, *Hanseniaspora uvarum* was more or less attractive than *Saccharomyces cerevisiae* to the spotted wing drosophilid *Drosophila suzukii* (Lasa et al. 2019), implying that microbe-insect interactions can be context-dependent. This interpretation may also explain why seemingly ubiquitous microbes, such as *Staphylococcus epidermidis,* guide insects to different resources (Cusumano et al. 2022).

Insect-microbe interactions extend beyond insect foraging. The yeast *H. uvarum* induces both upwind flight and oviposition by *D. suzukii* adults, and attracts *D. suzukii* larvae (Chakraborty et al. 2022), likely because *H. uvarum* is a food source that supports

larval development and outcompetes potentially dangerous microbes in larval development substrates (Chakraborty et al. 2022). Similarly, females of the fungus gnat, *Bradysia impatiens* (Diptera: Sciaridae), are attracted to, and preferentially oviposit on, various species of *Pythium* fungi which infect geranium seedlings (Braun et al. 2012). *Pythium* fungi also attract the larvae of *B. impatiens* and serve as their food source (Braun et al. 2012). Indeed, there are many studies and reviews in the literature that describe intricate relationships between microbes and insects (Davis et al. 2013) in the contexts of herbivory (Grunseich et al. 2020), pollination (Cullen et al. 2021), floral cues (Crowley-Gall et al. 2021), forensics (Jordan & Tomberlin, 2017), and pest management (Hamby & Becher, 2016).

1.5.2) Host location by blood-feeding insects

Blood-feeding insects utilize a variety of cues to locate hosts. CO_2 is an important attractant for mosquitoes (Takken 1991), and mutant mosquitoes – being unable to sense CO_2 – also fail to respond to other host cues such as heat (McMeniman et al. 2014). A synthetic odor blend that included – among others – ammonia, lactic acid and CO_2 attracted more *Anopheles gambiae* and *A. arabiensis* (Diptera: Culicidae) mosquitoes than CO_2 alone (Busula et al. 2015). Black traps captured more yellow fever mosquitoes, *Aedes aegypti* (Diptera: Culicidae), than traps that were black- and white-striped, black- and white-patched, or white, suggesting that solid dark colors are important for host location in mosquitoes (Tang et al. 2021). *Aedes aegypti* females prefer heat sources with host-like temperature (34 °C) to those near ambient (20 °C) or potentially harmful (50 °C) (Zermoglio et al. 2017). Lastly, female *A. aegypti* use a combination of CO_2, dark contrasting colors, and heat to locate and recognize suitable hosts (Liu & Vosshall 2019).

Many blood-feeding insects exploit host cues to locate their blood hosts. The kissing bugs *Triatoma dimidiate* and *Rhodnius prolixus* (Hemiptera: Reduviidae) are attracted to CO_2 and a range of host-like temperatures, either of which enhance the attractiveness of semiochemical cues (Milne et al. 2009). Furthermore, in the presence of host odorants, *R. prolixus* nymphs leave their shelter, presumably initiating host foraging (Ferreira et al. 2019). The kissing bug *T. rubida* (Hemiptera: Reduviidae) prefers particular levels of

CO_2, relative humidity, and specific wavelengths of light (Indacochea et al. 2017). Common bed bugs, *Cimex lectularius* (Hemiptera: Cimicidae), orient towards heat sources at distances < 30 mm (DeVries et al. 2016), with other cues such as CO_2 mediating long-range attraction (Anderson et al. 2009). Chicken mites, *Dermanyssus gallinae* (Mesostigmata: Dermanyssidae), are activated by heat, particularly when their last blood meal has been digested (8–10 days after blood-feeding), and when they are likely in host-foraging mode (Kilpinen & Mullens 2004). The black fly *Simulium annulus* (Diptera: Simuliidae) is attracted to semiochemical and visual cues originating from the wings of its host, the common loon, *Gavia immer,* even when wings are presented on artificial surfaces (Weinandt et al. 2012). Horn flies, *Haematobia irritans* (Diptera: Muscidae), as severe cattle pests differentially land on more susceptible cows which differ in semiochemical, thermal, and/or visual cues from their less susceptible counterparts (Jensen et al. 2004; Basiel et al. 2021).

1.5.3) Host location mediated by microbes

The common human skin bacteria *Staphylococcus epidermidis*, *Corynebacterium minutissimum*, and *Bacillus subtilis* emit odorants that attract *Anopheles gambiae* mosquitoes (Verhulst et al. 2010). Skin microbiota differ among humans and thus affect their relative attractiveness to mosquitoes (Showering et al. 2022). Humans most attractive to *A. gambiae* have high densities of skin microbes and a great abundance of *Staphylococcus* spp., suggesting that *Staphylococcus* spp. contribute to the attractiveness of humans to mosquitoes (Verhulst et al. 2011). As microbes produce species- or strain-specific odor blends (Green et al. 2014; Peach et al. 2021), it follows that the species composition of skin microbiomes also affects its odor profile and thus the attractiveness of humans to host-seeking insects. With the human skin microbiome known to affect mosquito attraction and host recognition (Verhulst et al. 2011), it is conceivable that the skin microbiomes of other vertebrates, such as cattle (Zinicola et al. 2015a), may also affect their attractiveness to blood-feeding insects including stable flies.

Microbial communities and their odor profiles, respectively, affect oviposition decisions of stable flies. Certain bacteria, namely *Pseudomonas* sp., *Citrobacter freundii*,

Serratia fanticola, *Bacilus pumilis*, *Proteus penneri*, *Providencia* sp., and *Enterococcus* sp., all induce oviposition in female stable flies, with *C. freundii* stimulating oviposition almost as effectively as natural substrate for larval development (Romero et al. 2006). Microbial volatiles appear to be involved in drawing flies towards oviposition sites and – upon arrival – in prompting oviposition. These types of microbial effects on stable fly behavior provide impetus to study other potentially microbe-mediated stable fly behavior, particularly during host foraging. Cattle skin microbiota have been extensively investigated (Winther et al. 2022) in the context of bacterial infections that cause diseases such as mastitis (Andrews et al. 2019; De Buck et al. 2021) and bovine digital dermatitis (Zinicola et al. 2015a,b; Nielsen et al. 2016; Espiritu et al. 2020; Caddey & De Buck 2021; Caddey et al. 2021) but potential effects of cattle skin microbes on attraction of stable flies to their cattle hosts has never been investigated. Thus, this is the research objective in Chapter 3 of my thesis.

1.6) Stable flies as vectors for bovine mastitis-causing *Staphylococcus aureus*

1.6.1) Hematophagous insects as microbe vectors

Insect vectors are responsible for transmission of many disease-causing pathogens. Siphonapterans (fleas) are vectors for the bacterium *Yersinia pestis*, the causative agent of Plague (Wimsatt & Biggins 2009). Kissing bugs, specifically *Triatoma infestans*, *Rhodnius prolixus*, and *Panstrongylus megistus* (Hemiptera: Reduviidae), transmit Chagas disease-causing *Trypanosoma cruzi* (Steverding 2014). Onchocerciasis (river blindness) is caused by the parasitic nematode *Onchocerca volvulus*, which is vectored by simuliid blackflies (Hougard et al. 1997). Tsetse flies, *Glossina* spp. (Diptera: Glossinidae), transmit *Trypanosoma brucei*, the causative agent of sleeping sickness (Malvy & Chappuis 2011). Mosquitoes alone transmit a plethora of pathogens of significant medical importance. *Aedes* spp. mosquitoes vector the viruses which cause Chikungunya (Lounibos & Kramer 2016), Dengue (Weetman et al. 2018), Rift Valley Fever (*Phlebovirus*) (Kwasnik et al. 2021), Yellow Fever (Barrett & Higgs 2007), and Zika (Rabaan et al. 2017), among others. *Culex* spp. mosquitoes transmit the viruses which cause Rift Valley Fever (Kwasnik et al. 2021), and West Nile Fever (Hayes 2001),

as well as the parasitic nematode *Wuchereria bancrofti* which causes lymphatic filariasis (Manguin et al. 2010). Finally, *Anopheles* spp. mosquitoes vector *W. bancrofti* and parasitic protists in the genus *Plasmodium*, the causative agents of Malaria (Manguin et al. 2010).

Adverse impacts of stable flies on feed efficiency, weight gain, and milk production of cows, and ultimately the 'bottom line' of life stock industries, have been well studied and appear to be mostly conclusive (Bruce & Decker 1958; Campbell et al. 1977; Campbell et al. 2001; Taylor et al. 2012; see also section 1.1 above). However, although the role of stable flies as vectors of disease-causing pathogens has been extensively studied, results are not always conclusive, particularly in earlier studies (Greenberg 1973). These studies have investigated the ability of stable flies to vector the pathogens which cause polio (Anderson & Frost 1912, 1913; Rosenau & Brues 1912; Sawyer & Herms 1913), paratyphoid (Birk 1932), Leishmaniasis (Berberian 1938; Lainson & Southgate 1965), Yellow Fever (Hoskins 1934), tularemia (Olsufiev 1940), African Swine Fever (Mellor et al. 1987) and even Plague and Plague-like diseases (Wayson 1914) but results were often inconclusive. More recent data on pathogen transmission by stable flies are more conclusive but conflicting reports still exist. When stable flies had ingested blood inoculated with *Enterobacter sakazakii* bacteria, they transmitted the bacteria to sources of sterile blood and honey-water for at least 20 days (Mramba et al. 2007). Stable flies that had fed on a hamster infected with the Rift Valley Fever virus at a biologically realistic viral load of $10^{9.7}$ plaque-forming units per mL, transmitted the virus to healthy hamsters in 57% of subsequent feeding bouts (Turell et al. 2010). Stable flies that sequentially fed on blood infected with West Nile Virus (WNV), and then on sterile blood, transmitted WNV and WNV-RNA in 6% and 26.5%, respectively, of all feeding trials (Doyle et al. 2011). When stable flies first fed on blood infected with the arterivirus that causes porcine reproductive and respiratory syndrome in pigs, and then fed on healthy pigs, they failed to transfer the arterivirus (Rochon et al. 2011). Moreover, stable flies were found to not carry the spiral-shaped bacterium *Treponema phagedenis* (Thibodeaux et al. 2021) which is deemed a causal agent of bovine digital dermatitis. Conversely, 11.3% of wild stable flies carried the Gram-negative bacterium *Anaplasma marginale*, the causative agent of bovine anaplasmosis (Araujo et al. 2021). Outbreaks of

viral lumpy skin disease in bovines were correlated with high abundance of stable flies, implying a vectorial function of flies (Kahana-Sutin et al. 2017). Finally, modelling transmission of the African Swine Fever virus on a pig farm revealed that increasing stable fly loads (from 5–10 to 50–100 flies per pig) increased viral transmission from 10–18% to 48–64% (Vergne et al. 2021).

1.6.2) Bovine mastitis

Bovine mastitis (henceforth 'mastitis') is a painful inflammation of the udder typically caused by bacterial infections. Depending upon the class of infection (clinical, sub-clinical or chronic), symptoms range from swollen udders, fevers, watery or clotted milk, to death (Cheng & Han 2020, and references therein). Regardless of infection class, infected cows commonly produce less milk, resulting in lost revenue of $177 USD per cow per year in Northwestern Europe and Canada (Hogeveen et al. 2019). In Ethiopia, revenue losses amounted to $29 per cow per year, but the impact was far higher when adjusted for relative incomes between regions. Approximately 58%, 26% and 17% of these losses were attributed to lower milk production, culling of afflicted cows, and veterinary costs, respectively (Hogeveen et al. 2019). In the US, mastitis-caused revenue losses were estimated to be $72 per cow per year which – when multiplied by the 8.7 million cows in the US – add up to $629 million (Hogeveen et al. 2019).

Infection with *Staphylococcus aureus* is a cause of mastitis (Zhao & Lacasse 2008). Besides the 'typical' mastitis symptoms (see above), infections of mammary tissues with specifically *S. aureus* also cause necrosis of milk-producing cells and their replacement with non-secretory cells (Zhao & Lacasse 2008), ultimately lowering milk production. As mastitis is caused by bacterial infection (Zhao & Lacasse 2008, and references therein), the mode of bacteria transmission ought to be investigated. In DNA fingerprint analyses for the presence of *S. aureus* bacteria, they were confirmed in samples from ectoparasitic horn flies, heifer mammary secretions, and heifer streak canals (where milk passes through the teat) (Gillespie et al. 1999). Noteworthy, the same two bacterial strains isolated from flies were found in all but three heifer samples, suggesting that horn flies transmit *S. aureus* to cows. Furthermore, when the teats of healthy cows were exposed to

horn flies carrying *S. aureus*, intra-mammary infections occurred in three out of four trials (Owens et al. 1998). Lastly, scabs of heifers naturally infected with *S. aureus* contained high concentrations of these bacteria (Owens et al. 1998). Altogether, these results provide incentive to explore whether not only horn flies, but also stable flies, transmit mastitis-causing *S. aureus* to their vertebrate blood hosts. This is the research objective in Chapter 4 of my thesis.

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Tables

Table 1.1 Table summarizing work done on stable fly semiochemical attractants.

Chemical/substrate	Type of stimulus (# of studies)	Bioassay setting (# of studies)	Type of response (# of studies)	References
CO_2	Host cue (6) Oviposition cue (2) Other/not specified (10)	Lab (7)	Attraction (2) Activation (5) Egg-laying (1)	Hoy, 1970; Gatehouse & Lewis, 1973; Vale, 1980; Gersabeck et al., 1982; Warnes & Finlayson, 1985a; b; Mihok et al., 1996; Schofield et al., 1997; Schofield & Brady, 1997; Mohamed-Ahmed & Mihok, 1999; Cilek, 1999; Alzogaray & Carlson, 2000; Torr et al., 2006; Jeanbourquin & Guerin, 2007; Beresford & Sutcliffe, 2012; Phasuk et al., 2016; Tunnakundacha et al., 2017; Hogsette & Kline, 2017
		Field (11)	Attraction (11) No effect (1)	
1-octen-3-ol	Host cue (5) Oviposition cue (1) Other/not specified (7)	Lab (5)	Attraction (1) Activation (1) Deactivation (1) No effect (2)	Holloway & Phelps, 1991; Mullens et al., 1995; Mihok et al., 1995; Mihok et al., 1996, 2007; Schofield et al., 1997; Schofield & Brady, 1997; Djiteye et al., 1998; Cilek, 1999; Alzogaray & Carlson, 2000; Tangtrakulwanich et al., 2015; Phasuk et al., 2016; Zhu et al., 2022
		Field (8)	Attraction (6) No effect (4)	
Acetone	Host cue (3) Other/not specified (4)	Lab (3)	Attraction (1) Activation (2)	Warnes & Finlayson, 1985b; Mihok et al., 1995; Schofield et al., 1997; Schofield & Brady, 1997; Djiteye et al., 1998; Cilek, 1999; Mihok et al., 2007
		Field (4)	Attraction (3) No effect (3)	
Pineapple residue	Oviposition cue (1)	Field (1)	Attraction (1)	Solorzano et al., 2015
Vinasse	Oviposition cue (2)	Lab (1)	Attraction (1)	Serra et al., 2017; Souza et al., 2021
		Field (2)	Attraction (2)	

3-phenyl-1-propanol, hydrocinnamaldehyde, cinnamyl alcohol	Floral foraging cues (1)	Field (1)	Attraction (1)	Hammack & Hesler, 1996
Fruit from *Piliostigma reticulatum* Flowers from: *Acacia albida*, *Ziziphus mauritiana*, and *Acacia macrostachya*	Floral foraging cues (1)	Field (1)	Attraction (1)	Mueller et al., 2012
Phenol	Oviposition cue (2) Other/not specified (1)	Lab (2)	Attraction (1) No effect (2)	Tangtrakulwanich et al., 2015; Zhu et al., 2016; Zhu et al., 2022
		Field (2)	Attraction (2)	
4-methylphenol	Other/not specified (3)	Field (3)	Attraction (1) No effect (2)	Holloway & Phelps, 1991; Cilek, 1999; Mihok et al., 2007
3-n-propylphenol	Other/not specified (3)	Field (3)	Attraction (1) No effect (2)	Holloway & Phelps, 1991; Cilek, 1999; Mihok et al., 2007
m-cresol	Oviposition cue (2) Other/not specified (4)	Lab (2)	Attraction (2) Repellency (1) No effect (1)	Djiteye et al., 1998; Tangtrakulwanich et al., 2015; Zhu et al., 2016, 2022; Baleba et al., 2019; Lehmann et al., 2023
		Field (6)	Attraction (4) No effect (2)	
p-cresol	Oviposition cue (2) Other/not specified (2)	Lab (3)	Attraction (2) Activation (1) Repellency (1) No effect (2)	Jeanbourquin & Guerin, 2007b; Tangtrakulwanich et al., 2015; Zhu et al., 2016; Zhu et al., 2022
		Field (2)	Attraction (2)	
Acetic acid	Other/not specified (1)	Field (1)	Repellency (1)	Vale, 1980
Lactic acid	Other/not specified (1)	Field (1)	No effect (1)	Mihok et al., 1995
Butanoic acid	Other/not specified (1)	Lab (1)	Attraction (1) Activation (1)	Jeanbourquin & Guerin, 2007b

Ammonia	Host cue (2)	Lab (2)	Probing (1) No probing (1)	Hopkins, 1964; Gatehouse, 1970
Bacteria (*Pseudomonas* sp., *Citrobacter freundii*, *Serratia fanticola*, *Bacilus pumilis*, *Proteus penneri*, *Providencia* sp., *Enterococcus* sp.)	Oviposition cue (1)	Lab (1)	Egg laying (1)	Romero et al., 2006
Female stable fly cuticular hydrocarbons	Sex pheromone (3)	Lab (3)	Attraction (1) Mating behaviour induction (2) No effect (1)	Muhammed et al., 1975; Uebel et al., 1975; Carlson & Mackley, 1985
Stable fly feces	Other/not specified (2)	Lab (1) Field (1)	Attraction (1) No effect (1)	Carlson et al., 2000; Mihok et al., 2007
Rumen digesta	Oviposition cue (1)	Lab (1)	Activation (1)	Jeanbourquin & Guerin, 2007b
Dimethyl trisulphide	Oviposition cue (1)	Lab (1)	Activation (1)	Jeanbourquin & Guerin, 2007b
Skatole	Oviposition cue (1)	Lab (1)	No effect (1)	Jeanbourquin & Guerin, 2007b
β-citronellene	Oviposition cue (1)	Lab (1) Field (1)	Egg laying (1) Attraction (1)	Baleba et al., 2019
Animal urine (cow, buffalo, waterbuck, camel) Animal feces (rhinoceros, elephant, hippopotamus)	Oviposition cue (1)	Field (1)	No effect (1)	Mihok et al., 1995
Human skin	Host cue (1)	Lab (1)	Attraction (1)	Alzogaray & Carlson, 2000
Human breath	Host cue (2)	Lab (2)	Attraction (2)	Alzogaray & Carlson, 2000; Warnes & Finlayson, 1985b

Table 1.2 Table summarizing electroantennogram and gas chromatographic-electroantennographic detection studies conducted on stable flies

Reference	Chemical(s) and/or substrate(s) found to induce positive antennal responses	Chemical(s) and/or substrate(s) found to induce negative antennal responses	Chemical(s) and/or substrate(s) found to be inactive
Warnes & Finlayson, 1986	CO_2[H] 1-octen-3-ol[H] acetone[H] human breath[H] cattle odors[H] fresh cow feces odors[O]	acetic acid[H]	
Schofield et al., 1995	1-octen3-ol[H] 3-methylphenol[H] octan-1-ol[H] 1-bromooctane[H] octan-3-ol[H] octanal[H] 2-octanone[H]		acetic acid[H] acetone[H]
Birkett et al., 2004	1-octen-3-ol m-cresol p-cresol naphthalene 4-methyl-2-nitrophenol 2-methoxyphenol (Z)-3-hexen-1-ol 2-heptanone propyl butanone 3-octanol 2-decanol 1-nonanol 6-methyl-5-hepten-2-one linalool citronellol		propylbenzene phenol o-cresol acenaphthene styrene decane undecane α-pinene camphene indole skatole N,N-diethyltoluamide propionic acid
Tangtrakulwanich et al., 2011	1-octen-3-ol[H] indole[O] phenol[O] p-cresol[O] dimethyl trisulphide[O] 2-heptanone acetic acid hexanoic acid butyric acid (more active in males than females)		

	isovaleric acid (more active in females than males)		
Jeanbourquin & Guerin, 2007a	1-octen-3-ol^{O} dimethyl trisulphide^{O} butanoic acid^{O} α-humulene^{O} acetophenone^{O} isovaleric acid^{O} borneol^{O} heptan-1-ol^{O} β-caryophyllene^{O} citronellene^{O} D-limonene^{O} β-caryophyllene^{O} β-cyclocitral^{O} α-humulene^{O}		
Jeanbourquin & Guerin, 2007b Note: only a selection of the tested chemicals is shown here	oct-1-en-3-ol^{O} dimethyl trisulphide^{O} β-cyclocitral^{O} oct-1-en-3-ol* dimethyl trisulphide* p-cresol*		
Serra et al., 2017	butanoic acid^{O} hexan-1-ol^{O} pentanoic acid^{O} heptan-1-ol^{O} hexanoic acid^{O} 2-methoxyphenol^{O} 3-methoxyphenol^{O} 4-methoxyphenol^{O} phenylethyl alcohol^{O} 4-ethylbenzaldehyde^{O} acetophenone^{O} 2,6-dimethyl-7-octen-2-ol^{O} cinnamic aldehyde^{O} 3-methylbutanoic acid^{O} phenol^{O} naphthalene^{O} cymen-7-ol^{O}		

H-chemical/substrate tested as a host cue
O-chemical/substrate tested as an oviposition cue
*-synthetic analogue of chemical

Chapter 2: Abiotic characteristics and organic constituents of oviposition attractants and stimulants for gravid female stable flies, *Stomoxys calcitrans*

A near identical version of this chapter has been published in *Entomologia Experimentalis et Applicata* with the following authors: Saif Nayani, Regine Gries, Adam Blake & Gerhard Gries. doi: 10.1111/eea.13390

Abstract

Gravid female stable flies, *Stomoxys calcitrans* (Diptera: Muscidae), oviposit in many types of organic substrates, including animal feces, but there is limited information as to which factors mediate attraction and oviposition. Here, we (1) tested effects of oviposition site moisture and odor on attraction and oviposition by flies, and (2) selected a highly effective oviposition site (fly rearing medium) to determine the key constituent(s) that mediate(s) attraction and oviposition. In moving- and still-air olfactometers as well as large laboratory rooms, we show that (1) odor and moisture of oviposition sites play distinguishable functional roles in the close-range attraction of gravid female flies and their propensity to oviposit, (2) rearing medium containing fish food, wheat bran, wood chips, and a watery solution of ammonium bicarbonate [$(NH_4)HCO_3$, releasing NH_3 and CO_2] is a more appealing oviposition site to female flies than is cow feces, (3) ammonium bicarbonate in this medium is the key constituent for stable fly attraction and oviposition, (4) NH_3 alone or in combination with CO_2, but not CO_2 alone, attracts stable flies and induces oviposition, and (5) NH_3/CO_2 and fish food in combination are more attractive than NH_3/CO_2 or fish food alone. With fecal bacteria reportedly emitting NH_3, and with stable fly larval development reportedly reliant on (fecal) microbes, it follows that gravid female flies may be guided by airborne microbe-derived cues originating from prospective oviposition sites. Isolating these microbes and identifying their odorants could enable the development of a synthetic odor blend which, coupled with NH_3 and CO_2, may prove highly effective as a trap lure to capture gravid female stable flies.

Keywords: long-range attraction, egg-laying, fecal odor, fish food odor, moisture, fly rearing medium, ammonium, carbon dioxide, Diptera, Muscidae, long-range foraging cue, egg-laying site

2.1) Introduction

Oviposition sites and strategies of flies (Diptera) are diverse and guild-specific, with microbial, chemical (olfactory), and visual cues mediating attraction of gravid female flies and affecting their oviposition decisions (Lam et al. 2007; Joseph et al. 2009; Brodie et al. 2014; Sumethasorn & Turner 2016; Hoshizaki et al. 2020).

Stable flies, *Stomoxys calcitrans* (Muscidae), are blood feeding pests of cattle (Wood 1985). Females lay eggs and larvae develop in many different substrates (Machtinger et al. 2014), including decomposing and fermenting organic matter (Lysyk 1993; Berkebile et al. 1994; Taylor & Berkebile 2011; Cook et al. 2018), rotting vegetation (Solorzano et al. 2015; Serra et al. 2017), grass clippings (Ware 1966), spilled cattle feed (Meyer & Peterson 1983), silage mounts (Lysyk 1993) as well as, in North America, cow and horse feces (Albuquerque & Zurek 2014; Machtinger et al. 2014), and, in Africa, donkey and sheep feces (Baleba et al. 2019a). In preliminary observations, we noticed that the medium used for rearing stable fly larvae (henceforth 'fly rearing medium'), containing wheat bran, fish food, ammonium bicarbonate [$(NH_4)HCO_3$], wood chips, and water as constituents (Friesen et al. 2018), seemed more appealing as an oviposition resource to adult stable flies than cow feces, an excellent oviposition site for stable flies (Meyer & Petersen 1983; Foil & Hogsette 1994).

Oviposition site selections by flies, in general, seem to be based not only on intrinsic properties (e.g., nutritional value) of oviposition resources that are optimal for offspring development – analogous to the preference–performance hypothesis originally developed for insect herbivores (Gripenberg et al. 2010) – but are based also on biotic factors that indicate future larval competition – social-information-use hypothesis (Grüter & Leadbeater 2014) – predation, or parasitism. For example, the presence of conspecific eggs stimulates oviposition by female sand flies, *Lutzomyia longipalpis* (Elnaiem & Ward 1991) due to an oviposition pheromone, dodecanoic acid (Dougherty et al. 1994; Dougherty & Hamilton 1997), which is deposited onto eggs during oviposition (Dougherty et al. 1992). The presence of conspecific eggs also informs oviposition decisions by female house flies, *Musca domestica* (Lam et al. 2007). Similarly, mosquitoes tend to oviposit in habitats with conspecific larvae as indicators of habitat suitability (Blaustein & Kotler 1993; Allan & Kline 1998; Mwingira et al. 2019) but

avoid oviposition in sites occupied by predators or resource competitors (Kiflawi et al. 2003; Blaustein et al. 2004). Gravid female sand flies *Phlebotomus papatasi* are strongly attracted to rearing medium containing active larval stages (Marayati et al. 2015), whereas gravid female stable flies avoid ovipositing in substrates occupied by conspecific larvae, house fly larvae, and the mite *Macrocheles muscaedomesticae* (Baleba et al. 2020). The presence of mites reduced the hatchability of fly eggs and survival of fly larvae, and the presence of con- and heterospecific resource competitors – density dependently – reduced the body weight of larvae, pupae, and adults, and the percent emergence of adults (Baleba et al. 2020). The feeding experience of insect larvae can affect their oviposition choice as adults (e.g., Shikano & Ismans 2009), but adult stable flies do not necessarily oviposit in the same type of substrate in which they developed as larvae (Baleba et al. 2019b). The propensity of flies to oviposit can be reduced by treating oviposition substrate with catnip oil or its major nepetalactone constituents (Zhu et al. 2012).

Oviposition site selection by flies can be viewed as a two-stage process, similar to what has been described for mosquitoes (Eneh et al. 2016). Site location starts from a long range mediated primarily by visual and/or olfactory 'oviposition attractants' (conversely to 'repellents'; Clements 1999) that prompt gravid female flies to engage in oriented flight toward the oviposition substrate. At close range, when flies are near or at the resource, 'oviposition stimulants' (conversely to 'deterrents'; Clements 1999), such as semi-volatile semiochemicals as well as gustatory and tactile cues, prompt oviposition by flies.

As stable flies lay eggs in a wide range of oviposition substrates, it is likely that all these substrates have common characteristics, such as specific moisture content and/or odor profile. Relative moisture content of oviposition sites was correlated with both the abundance of stable fly larvae (Friesen et al. 2016) and the number of adult flies emerging from oviposition sites (Wienhold & Taylor 2012). Similarly, during periods of increased rainfall more stable fly adults were present (Mullens & Peterson 2005). Odorants such as dimethyl trisulfide, butanoic acid, and *p*-cresol were found in headspace volatiles of both cow and horse feces, and were shown to elicit responses from stable fly antennae (Jeanbourquin & Guerin 2007). As these compounds are generic decomposition

products, they are likely also released from decomposing organic materials. The attraction of stable flies to donkey and sheep feces in Africa is mediated by β-citronellene and carvone (Baleba et al. 2019a).

The relative contribution of oviposition site moisture and odor on oviposition site selection by gravid female stable flies, and many other flies, is still not known. Moreover, if fly rearing medium were indeed superior to animal feces as a fly oviposition site, then fly rearing medium combined with a lethal agent could be developed to attract gravid female flies and kill their offspring to curtail fly populations in life stock production facilities. As a step toward this goal, it would also be important to determine the key constituents of the fly rearing medium that attract flies and stimulate oviposition. Therefore, our objectives (O) were to: (O1a/b) determine the effects of oviposition site moisture and odor as oviposition attractants (a) and as oviposition stimulants (b) for stable flies, (O2) compare the ability of cow feces and 'fly rearing medium' to attract flies and stimulate oviposition, and (O3) determine which constituents of 'fly rearing medium' – as a predicted superior oviposition site – attract flies and stimulate oviposition.

2.2) Materials and methods

2.2.1) Rearing of stable flies

Flies were kept in a hyperbolic growth chamber (BioChambers, Winnipeg, MB, Canada) located on the Burnaby campus of Simon Fraser University. The chamber was kept at 26 °C and a L14:D10 h photocycle. Adult flies were housed in wire mesh cages (46 × 46 × 46 cm; BioQuip, Rancho Dominguez, CA, USA) and fed twice a day with citrated bovine blood obtained from a local slaughterhouse. Blood-soaked cotton pads (Fluffs, Montreal, QC, Canada) were placed on the tops of cages, allowing the flies to feed on the blood through the mesh. At least 3× per week, oviposition was induced by placing pieces of wet black cloth (20 × 8 cm) as oviposition sites on top of cages, enabling oviposition by flies on the undersides of the cloths through the mesh. Eggs were then removed from the cloths and transferred to a rearing medium containing wood chips (200 g; Hyon Bedding, Prince George, BC, Canada), wheat bran (500 g; Rogers Foods, Armstrong, BC, Canada), staple fish food (115 g; Nutrafin, Montreal, QC, Canada), and ammonium bicarbonate

(50 g; Oakwood Chemical, Estill, SC, USA) dissolved in 1600–2000 mL of water (Friesen et al. 2018). Over a 2-week period, first instars hatched and developed through second and third instars to the pupal stage, which lasted 4–7 days. Pupae were then manually transferred to wire mesh cages (see above).

For bioassays, cold-sedated male and female flies were separated based on sex-specific characteristics, with males having a larger dark spot at the base of their abdomen than females, and females protruding their ovipositor when their abdomen is gently squeezed with forceps.

2.2.2) O1(a): Effects of oviposition site moisture and odor on fly attraction

Attraction of flies to oviposition sites was tested in a custom-built, moving-air Pyrex glass olfactometer (Figure 2.1A). Using a pump (Gast, Benton Harbor, MI, USA), air was drawn at 1 mL min^{-1} through each of two stimulus chambers (Figure 2.1A; 3a, 3b) interconnected to fly interception chambers (Figure 2.1A; 7a, 7b) via male–female joints (Figure 2.1A; 6a, 6b). These interception chambers allowed fly entry, but not exit, from the central chamber (‘1’ in Figure 2.1A). During bioassays, flies could sense the odor and/or moisture of test stimuli but could not see or contact test stimuli. Light-exposing only the lower part of the central chamber, where the two air streams entered, increased the number of (phototactically positive) responding flies. For each bioassay replicate, 20 fed gravid females were introduced into the central chamber and allowed 24 h to respond to test stimuli. Then, the olfactometer was placed into a walk-in freezer (–15 °C) to cold-euthanize all flies and to count those in each interception chamber. Flies remaining in the central chamber were deemed non-responders.

To test the effect of oviposition site moisture on attraction of flies, a dry or a wet paper towel (Scott, Philadelphia, PA, USA) was inserted into a stimulus chamber. For the wet towel, water was added to the point of runoff. Whereas the air drawn over the dry paper towel had a relative humidity identical to that in the laboratory (40–50%), the air drawn over the wet paper towel had a humility of 85–95%.

To test the effect of oviposition site odor (and moisture; see below) on attraction of flies, an inverted bottle cap (2.5 × 1 cm) was baited with cow feces (5 g), or left unbaited (control), and placed in a stimulus chamber. After collection, cow feces was stored in a –

15 °C freezer to maintain freshness, and was thawed for bioassays. Its moisture content was determined to be 90.12 ± 2.49% in all experiments (see below). Two-choice experiments 1–6 then tested all possible stimulus pairs in a full factorial design (Table 2.1), as follows: wet vs. dry (Exp. 1), wet & feces vs. dry & feces (Exp. 2), wet & feces vs. wet (Exp. 3), dry & feces vs. dry (Exp. 4), dry & feces vs. wet (Exp. 5), and wet & feces vs. dry (Exp. 6). Because completely dry feces with zero moisture content is virtually odorless and would not have allowed us to test the effect of feces odor on behavioral responses of flies, feces was not dried prior to testing in bioassays but it did dry during the course of 24-h replicates. The fact that we did find an interaction between feces and moisture (see Results) supports the conclusion that the moisture content of feces was insufficient to account for moisture as an abiotic test stimulus.

To determine the dry weight and moisture content of cow feces that was tested in experiments, 10-g samples (n = 5) of feces were placed on separate glass Pyrex Petri dishes (ca. 9 cm diameter) and heated on a hot plate (Corning, New York, NY, USA) under a fume hood. Dishes were re-weighed every 2 min until three consecutive measurements revealed no further weight loss. Final dry weights were then subtracted from initial weights, with the percent weight-differential indicating moisture content (90.12 ± 2.49%).

2.2.2) O1(b): Effects of oviposition site moisture and odor as oviposition stimulants

Egg-laying by flies in response to both the moisture content and odor of oviposition sites was tested in mesh cages (46 × 46 × 46 cm) fitted with a jar (4 × 6 cm; Figure 2.1B; 13) containing a water-soaked cotton wick (Richmond, Charlotte, NC, USA) as a water source for bioassay flies. To test the effect of moisture on oviposition, two stimulus jars (each 5.5 × 7 cm; Figure 2.1B; 8a, 8b), one of which was filled two-thirds with water and the other left empty, were placed 30 cm apart from each other in the cage (Figure 2.1B). A one-third portion of a piece of black cloth (20 × 8 cm; Figure 2.1B; 10) was lowered into each jar and the remaining two-third portion was secured with a rubber band to form a taut surface over the jar opening and to spill over the jar rim (Figure 2.1B; 8-10; as in Friesen et al. 2018). Water in a jar was drawn up by the cloth moistening its entire surface. To test the effect of odor on egg laying, an inverted bottle-cap (2.5 × 1 cm;

Figure 2.1B; 12) was baited with cow feces (5 g), or left empty (control), and placed on the taut cloth covering the opening of each jar. The remaining portion of the cloth spilling over the jar rim was then folded back over the bottle cap (Figure 2.1B; 9, 11).

For each bioassay replicate, 20 gravid female flies were released into a cage and allowed 24 h to lay eggs. The flies were then cold-euthanized, and the eggs they had laid on cloth surfaces, bottle caps, and feces (if applicable) were counted. Two-choice experiments 7–12 tested all possible stimulus pairs in a full factorial design (Table 2.1), as follows: wet vs. dry (Exp. 7), wet & feces vs. dry & feces (Exp. 8), wet & feces vs. wet (Exp. 9), dry & feces vs. dry (Exp. 10), wet vs. dry & feces (Exp. 11), and wet & feces vs. dry (Exp. 12).

2.2.3) O2: Comparative ability of cow feces and fly rearing medium to attract and induce oviposition by flies

Based on preliminary observations, the medium used for rearing stable fly larvae seemed more appealing as an oviposition resource to adult stable flies than cow feces, which reportedly is an excellent oviposition site for stable flies (Meyer & Petersen 1983; Foil & Hogsette 1994). To investigate this observation experimentally, we tested the ability of the fly rearing medium to attract and induce oviposition by flies (Table 2.1; Exp. 13). For this experiment, two jars (5.5 × 7 cm; Figure 2.1B; 9a, 9b), each containing a piece of water-soaked black cloth that was secured at, and spilled over, the jar rim (Figure 2.1B), were placed in the lateral chambers of a 3-chamber still-air olfactometer (Figure 2.1C; 15a, 15b). In each replicate, an inverted bottle-cap (2.5 × 1 cm; Figure 2.1B; 12) was placed on the taut cloth covering the opening of each jar. By random assignment, one cap was baited with 5 g of larval rearing media, whereas the other was kept empty (control). Then, the portion of the cloth that spilled over the jar rim was folded back over the cap (Figure 2.1B; 8–11). The 3-chamber olfactometer allowed us to score both attraction and oviposition by flies in the same bioassay. In each replicate of experiment 13 (n = 12), oviposition sites were placed into lateral chambers and 12 mated female flies were released into the central chamber (Figure 2.1C; 14). Sensing the presence of oviposition sites, flies entered the lateral chambers though a tapered mesh funnel (Figure 2.1C; 16) which allowed entry but not exit of flies. Bioassays were terminated after 24 h by cold-

euthanizing the flies and by counting the flies in each chamber (attraction) and the eggs (oviposition stimulation) on each of the two oviposition sites.

To determine whether fly rearing medium was indeed more effective than cow feces for attraction and oviposition by stable flies, we also tested fly rearing medium and cow feces head-to-head (Table 2.1; Exp. 14). We prepared oviposition sites (rearing medium and cow feces) as described (Exp. 13), and ran experimental replicates in the 3-chamber olfactometer following the established protocol (Exp. 13).

2.2.4) O3(a): Constituent(s) of rearing medium as oviposition attractants and stimulants

With evidence that the fly rearing medium was superior to cow feces for attraction and oviposition by flies (see Results), we proceeded to determine the key constituents of fly rearing medium that mediated the flies' responses. To this end, we tested the complete rearing medium with all its constituents (wood chips, fish food, wheat bran, ammonium bicarbonate) vs. a partial rearing medium from which one constituent at a time had been deleted (Table 2.1), such as wood chips (Exp. 15), fish food (Exp. 16), wheat bran (Exp. 17), and ammonium bicarbonate (Exp. 18). This type of 'subtractive' method is the most efficient to determine the key attractants/constituents of a resource (Byers, 1992). Release of ammonium (NH_3) and carbon dioxide (CO_2) from the medium was measured using a MultiRAE Wireless Portable Six-Gas Monitor (Honeywell, Charlotte, NC, USA) and a Q-Track Indoor Air Quality Monitor (TSI, Shoreview, MN, USA). For each of experiments 15–18, both the complete and the partial rearing media were prepared at 5-g equivalents and the responses of flies, both attraction and oviposition stimulation, were tested in 3-chamber still-air olfactometers following the protocol of experiment 13.

2.2.4) O3(b): Effects of NH_3 and/or CO_2 as oviposition attractants and stimulants

With evidence that the salt ammonium bicarbonate was a key constituent of the rearing medium for attraction and ultimately oviposition by flies (see Results), it was important to determine which of the two gases, NH_3 or CO_2, that are released from ammonium bicarbonate mediated the responses of flies. As we could not selectively manipulate the release of either one of these two gases from ammonium bicarbonate, we opted to test the

effect of sodium bicarbonate ($NaHCO_3$, which releases only CO_2) and ammonium bicarbonate in two parallel experiments (experiments 19 and 20, respectively). Test stimuli were prepared by dissolving sodium bicarbonate (5 g) – or ammonium bicarbonate (5 g) – and sugar (25 g) in 20 mL of water. Sugar was added to obtain a more viscous solution which seemed to facilitate sustained release of gases. The responses of flies, both attraction and oviposition stimulation, were tested in 3-chamber olfactometers (Figure 2.1C) following the protocol of experiment 13.

2.2.4) O3(c): Interactive effects between fish food odor and NH_3/CO_2 on fly attraction

Although the fish food odor of fly rearing medium failed to attract stable flies in 3-chamber olfactometer bioassays (see Results), we wanted to investigate potential interactions between fish food odor and NH_3/CO_2 for fly attraction on a larger scale. We chose fish food, rather than wheat bran or woodchips, for testing potential interactions with NH_3/CO_2 because wheat bran odor proved repellent to flies (see Results), and the terpene odor of wood chips is not indicative of a nutrient source. To this end, we ran bioassays in a large insectary room (225 × 230 × 230 cm) lit by a combination of plant illumination lights (Standard Products, Saint-Laurent, CA, USA) and day lights (Philips, Amsterdam, The Netherlands) with a L15:D9 photoperiod. Paired stimulus jars (Figure 2.1) fitted with inverted bottle caps (6 × 1 cm) holding test stimuli (see below) were placed 2 m apart in the room. Cylinders (28 × 9.5 cm) of black cardstock (ArtSkills, Bethlehem, PA, USA) (Figure 2.1D; 17) coated on their inner surface with adhesive (Tanglefoot, Marysville, OH, USA) were placed vertically around the jars to capture flies that landed on either stimulus (see below) to oviposit. Twenty-four h after 20 flies were released into the room, the cylinders and jars were removed and the flies captured were counted. Experiments 21–24 (Table 2.1) offered flies choices between (1) fish food vs. water (control) (Exp. 21), (2) NH_3/CO_2 vs. water (Exp. 22), (3, 4) fish food + NH_3/CO_2 vs. either NH_3/CO_2 (Exp. 23) or fish food (Exp. 24). NH_3 and CO_2 were released from ammonium bicarbonate (5 g) dissolved in water (150 mL).

2.2.5) Statistical analysis

Statistics were run with RStudio v.4.1.1 (Rstudio Team 2022). Data of each experiment were analyzed with binomial generalized linear models (BGLMs) using quasibinomial errors to account for overdispersion (Crawley 2007; Nayani et al. 2022; R Core Team 2022). These analyses compared an intercept-only model to a null model, where the intercept was assumed to be 0.5 (logit of 0), with a likelihood ratio test to determine whether the proportions of (1) flies being attracted to treatment stimuli (Exps. 1–6 and 13–24), or (2) total eggs laid on treatment stimuli (Exps. 7–20), differed from a hypothetical 0.5 proportion. To test for differences in proportions among experiments sharing a common stimulus, we created similar generalized linear models with data from multiple experiments. We compared models with an individual intercept for each experiment to a model with a single intercept, again with a likelihood ratio test. When a significant difference between experiments was observed, the data in these experiments were compared with post-hoc all-pairwise contrasts for differences in proportions of flies attracted to – and eggs laid on – the various treatment stimuli (Hothorn et al. 2008; Nayani et al. 2022). A significance threshold of $\alpha = 0.05$ was considered in all experiments.

2.3) Results

2.3.1) O1(a): Effects of oviposition site moisture and odor as oviposition attractants

Each of oviposition site moisture, odor, as well as moisture and odor combined, attracted more flies than a dry (blank) control (Figure 2.2, Table 2.2). None of these three stimuli was superior in attracting flies relative to a dry control (likelihood ratio test: $F = 0.074$, d.f. = 1, $P > 0.05$). Tested head-to-head, the odor of oviposition sites was not more attractive than the moisture. However, odor and moisture in combination attracted more flies than moisture alone but not more flies than odor alone, implying a superior role of odor as an oviposition attractant for gravid female flies.

2.3.1) O1(b): Effects of oviposition site moisture and odor as oviposition stimulants

Oviposition sites that were moist, odorous, or both invariably received all eggs when tested against a dry (blank) control (Figure 2.3, Table 2.2). Consequently, these three

stimuli could not, statistically, be compared to determine differences between them. Tested head-to-head, moist sites received proportionately more eggs than odorous sites, and sites that were both moist and odorous received more eggs than sites that were either moist or odorous. Combined, these data imply that moisture is a relatively more important oviposition stimulant to flies than odor.

2.3.2) O2: Comparative ability of cow feces and fly rearing medium to attract flies and induce oviposition

Fly rearing medium proportionally attracted more flies and received more eggs than corresponding unbaited controls (Figure 2.4). When rearing medium and cow feces as oviposition sites were tested head-to-head, rearing medium proportionally attracted more flies and received more eggs than cow feces (Figure 2.4).

2.3.3) O3(a): Constituent(s) of rearing medium as oviposition attractants and stimulants

As fly rearing medium was more effective than cow feces for attraction and oviposition by flies (Figure 2.4), we proceeded to determine the key constituent(s) of rearing medium that mediated attraction and egg laying by flies (Figure 2.5). Neither woodchips nor fish food as medium constituents had any effect on attraction or oviposition by flies. Wheat bran, surprisingly, had an adverse effect on responses of flies. Rearing medium without wheat bran proportionally attracted more flies and received more eggs than medium with wheat bran (Figure 2.5). Ammonium bicarbonate (releasing NH_3 and CO_2) – as the key constituent of rearing medium – proportionally attracted more flies and received more eggs than medium without it. Relative to the complete rearing medium, medium lacking ammonium bicarbonate attracted fewer flies, and received fewer eggs, than medium lacking any of the other constituents.

2.3.3) O3(b): Effect of NH_3 and/or CO_2 as oviposition attractants and stimulants

CO_2 released from the watery solution of sodium bicarbonate attracted as few flies and induced as little oviposition as the water control (Figure 2.6). Conversely, NH_3 and CO_2 released from the watery solution of ammonium bicarbonate attracted more flies and

induced more oviposition than the water control. The ammonium bicarbonate solution attracted more flies, and received more eggs, than the sodium bicarbonate solution.

2.3.3) O3(c): Interactive effects between fish food odor and NH_3/CO_2 on fly attraction

In insectary rooms, adhesive-coated traps (Figure 2.1D; 17) baited with fish food were as ineffective as a water control in attracting and capturing flies (Figure 2.7). Conversely, traps baited with the ammonium bicarbonate solution (releasing NH_3 and CO_2) captured significantly more flies than the water control. Traps baited with both fish food and ammonium bicarbonate captured significantly more flies than traps baited only with either ammonium bicarbonate or fish food alone (Exp. 24), revealing an interaction between fish food odorants and the gases NH_3/CO_2 on stable fly attraction.

2.4) Discussion

Our data support the following conclusions: (1) the odor and moisture content of oviposition sites, on their own or in combination, play key roles in attracting gravid female stable flies and prompting oviposition, (2) fly rearing medium is an oviposition resource superior to cow feces, attracting proportionately more flies and receiving more eggs, and (3) ammonium bicarbonate (emitting NH_3 and CO_2) is the key ingredient in fly rearing medium and interacts with fish food odorants for attraction and oviposition of flies.

2.4.1) Effects of oviposition site moisture and odor on fly attraction and oviposition

Testing, in a full factorial design, the effects of oviposition site odor (odor of cow feces with limited moisture content) and moisture (tested as a separate abiotic cue) on fly attraction and oviposition revealed that each of these two cues has an effect on fly behavior. Although odor and moisture, singly and in binary combination, attracted more flies and induced more egg-laying when tested against a dry and odorless (blank) control stimulus, there was no additive effect between odor and moisture on fly attraction and oviposition. However, testing odor and moisture head-to-head revealed a shift in the relative importance of these two cues during the sequential steps of locating oviposition

sites and initiating oviposition. Our findings that feces odor enhanced the effect of moisture, but moisture failed to enhance the effect of feces odor, on fly attraction, imply that odor may be a relatively more important oviposition attractant than moisture. Conversely, oviposition site moisture induced more oviposition by flies than feces odor, and moisture and odor in combination induced more oviposition than odor, suggesting that moisture is a relatively more important oviposition stimulant than odor. Selecting moist oviposition sites reduces the likelihood of larval desiccation and thus increases the reproductive fitness of ovipositing females. This conclusion is supported by previous reports that moist substrate allowed more fly maggots to complete their development to adults (Wienhold & Taylor 2012; Friesen et al. 2016). That females preferred moist oviposition sites with fecal odor to just moist sites, further indicates that females may also gauge nutrient availability for offspring development during oviposition decisions. Fecal semiochemicals can reflect the degree of fermentation that has taken place during digestion (Birkett et al. 1996; Mackintosh et al. 2002; Gilbery et al. 2010; Zapata et al. 2021; Liu et al. 2022), and likely change over time (Albuquerque & Zurek 2014), helping flies discern between fresh and aged sites, and select the latter for oviposition (Broce & Haas 1999).

2.4.2) Fly rearing medium is a superior oviposition site and ammonium bicarbonate is the key constituent

In North America, cow feces is a very appealing oviposition site for stable flies (Meyer & Petersen 1983). Similarly, cattle feces alone or mixed with soil, hay, or cattle feed, is a well-known medium for fly larval development in feedlots and dairies (Meyer & Petersen 1983). However, if there were any resources even more appealing to gravid females, these resources – coupled with lethal or hormonal agents – could be developed to attract and induce oviposition by gravid females and then suppress development of their larval offspring. As the fly rearing medium was very attractive to laboratory colony flies, and allowed many maggots to complete development to adults, we decided to test rearing medium experimentally for attraction and oviposition by flies. As rearing medium significantly attracted flies and prompted oviposition, we proceeded testing rearing medium head-to-head against cow feces. In this direct comparison, rearing medium

attracted 3.3× more flies and received 4.7× more eggs than cow feces, indicating that it was indeed superior to cow feces as an oviposition site. Future studies could investigate whether females preferentially ovipositing on rearing medium have higher reproductive fitness than females ovipositing on cow feces, in a manner reminiscent of the preference–performance hypothesis (Baleba et al. 2019a).

To determine the key constituents of the rearing medium that mediated attraction and oviposition by flies, we tested the rearing medium with all its constituents (wood chips, fish food, wheat bran, ammonium bicarbonate) vs. each of four partial rearing media lacking one of these constituents. Rearing medium with or without woodchips, and with or without fish food, were equally attractive to flies, indicating that neither of these two constituents had a behavior-modifying effect. Rearing medium without wheat bran was more attractive to flies and received more eggs than the complete medium, indicating that wheat bran, surprisingly, was repellent (Miller et al. 2009) to adult flies. Ammonium bicarbonate (releasing NH_3 and CO_2), in contrast, was the key ingredient in the rearing medium for both attraction and oviposition by flies. Medium with ammonium bicarbonate was 3.2× more attractive to flies and received 17.8× more eggs than medium without it. Similarly, among the four partial rearing media tested, the medium lacking ammonium bicarbonate attracted the fewest flies and received the fewest eggs, thereby confirming the key roles of NH_3 and CO_2 released from ammonium bicarbonate for fly attraction and oviposition.

With both NH_3 and CO_2 released from ammonium bicarbonate in moist rearing medium, either gas alone or both gases in combination could have triggered the responses of flies. As a first step to address the contributing role of these gases for fly attraction, we ran two parallel experiments, testing the effects of (1) sodium bicarbonate in water (releasing CO_2) vs. water, and (2) ammonium bicarbonate in water (releasing NH_3 and CO_2) vs. water. As CO_2 alone did not attract flies, but NH_3 with CO_2 did, it follows that the flies responded to NH_3 alone or in combination with CO_2. Whether, and to what extent, NH_3 alone attracts flies could be tested in two parallel follow-up experiments, one testing again ammonium bicarbonate in water (releasing NH_3 and CO_2) vs. water, and the other testing ammonium hydroxide (NH_4OH) in water (releasing NH_3) vs. water.

2.4.3) Interaction between fish food odorants and NH_3/CO_2

Fermentation of organic matter, such as animal feces, produces both odorants and gases (Birkett et al. 1996; Zapata et al. 2021; Liu et al. 2022). Fish food odor and NH_3/CO_2 in combination attracted 3–6× more flies than did NH_3/CO_2 or fish food odor alone, indicating an interaction between NH_3/CO_2 and fish food odor as oviposition attractants for gravid female stable flies. As the combined effect of NH_3/CO_2 and fish food odor on fly attraction was greater than the sum of their separate effects, the interaction can be considered (weakly) synergistic. Whether the presence of water in NH_3/CO_2 contributed to the weekly synergistic effect is yet to be investigated. That fish food odor on its own, unlike NH_3/CO_2 on its own, was not attractive to flies is surprising but reminiscent of analogous findings with ticks. CO_2 and odorants emitted from microbial symbionts of white-tailed deer, *Odocoileus virginianus*, synergistically attract Western black-legged ticks, *Ixodes pacificus*, but the odorants alone, unlike CO_2, are not attractive (Long et al. 2023). In our study, the distinctively different physicochemical characteristics of NH_3/CO_2 and fish food odorants may have guided stable flies at different stages during resource-foraging and/or may have complemented resource information. For example, CO_2 at elevated levels as an indicator of either host presence or fermenting organic materials in potential oviposition sites may have primarily activated foraging activities of stable flies, as shown in host-foraging mosquitoes (reviewed in Takken 1991) or may have been both an activator and an attractant, as shown in host-foraging bed bugs, *Cimex lectularius* (Anderson et al. 2009; Aak et al. 2014). Odorants of organic materials, such as fish food, may have enabled gravid female flies to distinguish between potential blood hosts and oviposition resources and to make informed oviposition decisions. Although fish food odor would not likely be encountered by gravid female flies seeking oviposition sites, and odor profiles of fish food and feces (preferred oviposition site) differ (data not shown), fish food odorants apparently indicated the presence of organic material. Analogous to mosquitoes which have unique CO_2 and semiochemical receptors that guide mosquitoes during various stages of host-foraging behaviour (van Breugel et al. 2015; Wooding et al. 2020; San Alberto et al. 2022), we predict that stable flies have distinct NH_3/CO_2 and odorant receptors that inform resource-foraging behavior.

2.4.4) Interpretation of findings and future directions

Our study adds to a growing body of literature reporting that semiohemical gases (NH_3/CO_2) alone or in combination with other semiochemicals attract dipterans, including blood-feeding stable flies and Tabanidae (Krčmar et al. 2010; Mihok & Lange 2012; Nayani et al. 2023), animal secretion-feeding canyon flies (Fannidae) (Mohr et al. 2011), fruit-feeding Tephritidae (Robacker & Warfield 1993; Robacker & Flath 1995; Liburd et al. 1998; Kendra et al. 2005; Yee 2007; Lasa & Williams 2021).

As NH_3 is produced through amino acid metabolism and the putrefaction of nitrogenous animal and plant matter (Yao et al. 2016), it can be a resource and nutrient indicator to foraging stable flies. When aging cattle manure became attractive to stable flies for oviposition, both NH_3 and CO_2 were consistently released from manure (Broce & Haas 1999), indicating that both gases could have guided oviposition site-seeking flies. As NH_3 is also produced by (fecal) bacteria (Macfarlane et al. 1986; Geypens et al. 1997; Geboes et al. 2006; Richardson et al. 2013; Yao et al. 2016), NH_3 could signal to foraging flies the presence of microbes in prospective oviposition sites. Various bacterial strains, including *Citrobacter freundii* and *Serratia fonticola* that were isolated from natural oviposition sites of stable flies and then grown on agar, induced oviposition behavior by stable flies (Romero et al. 2006). These bacteria not only provided foraging and oviposition cues for gravid female flies, but they are also essential for stable fly larval development. Stable fly larvae failed to develop in sterilized natural substrate composed of hay and horse manure and in sterilized trypticase soy egg yolk agar (TSEYA) but developed well in unsterilized natural substrate and in TSEYA inoculated with live *C. freundii* and *S. fonticola* (Romero et al. 2006). These stable fly larvae likely required live microbes, or their metabolites (e.g., vitamins and sterols), to complete development to the pupal stage (Brookes & Fraenkel 1958; Schmidtmann & Martin 1992; Watson et al. 1993).

With fecal bacteria emitting NH_3 (Richardson et al. 2013), and with stable fly larval development reliant on (fecal) microbes (Romero et al. 2006), it follows that gravid female flies are likely guided by airborne microbe-derived cues originating from prospective oviposition sites. Ammonium bicarbonate in rearing medium, releasing NH_3 and CO_2 and strongly attracting flies, may have substituted for the presence of NH_3- and

CO_2-emitting microbes. Similarly, fish food apparently substituted for the presence of other organic matter that flies typically seek for oviposition. To determine the semiochemicals that enhanced the attractiveness of NH_3/CO_2, headspace volatile extract of fish food could be analyzed by coupled gas chromatographic-electroantennographic detection (Gries et al. 2002), and those compounds eliciting responses from fly antennae could be tested alone or in combination with NH_3/CO_2 for attraction of flies. If any of the fish food odorants elicits antennal responses from flies it may even be produced by the very same microbes that emit NH_3 and CO_2. There are certainly many odorants that are emitted by metabolizing microbes capable of inducing responses in insects (Stensmyr et al. 2012; Uriel et al. 2020; Peach et al. 2021).

In future studies, we plan to run comparative bioassays of antennally active fish food and feces odorants for attraction of flies, and to isolate microbes from animal feces or decaying organic substrate to study their odor profiles, similar to research on sand flies (Marayati et al. 2015; Kakumanu et al. 2021). A synthetic blend of behaviorally active odorants coupled with both NH_3/CO_2 and a high moisture content of oviposition substrate, may prove highly effective as a trap lure to capture gravid female stable flies.

2.5) Acknowledgments

We thank two anonymous reviewers for constructive comments, Stephen Takács for graphical illustrations in Figure 2.1 and the graphical abstract, and Sharon Oliver for some word processing and comments. SN was supported by the Thelma Finlayson Graduate Entrance Scholarship, the Mutual Fire Insurance Company of British Columbia Graduate Scholarship, and by a Simon Fraser University Graduate Fellowship. Further funding for this project was provided by a Natural Sciences and Engineering Council of Canada (NSERC) – Industrial Research Chair to GG, with BASF Canada and Scotts Canada as industrial sponsors.

2.6) Availability of data and materials

Data and code are available from Mendeley Data: https://doi.org/10.17632/7h57yn2cwb.1 (link to dataset, Nayani et al. 2022).

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2.8) Figures and Tables

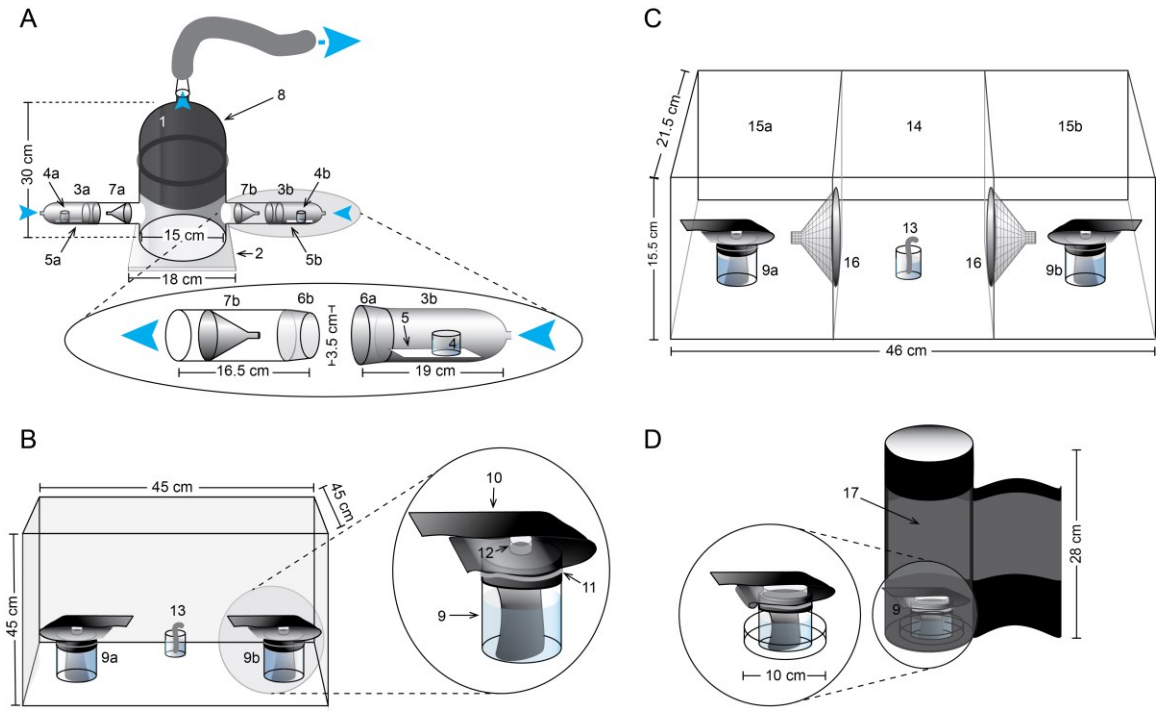

Figure 2.1 Graphical illustrations (not to scale) of the various experimental designs. (A) Moving-air olfactometer consisting of a custom-built Pyrex glass dome (1) residing on a glass plate (2). Air, drawn by a vacuum pump, entered the olfactometer through each of two stimulus chambers (3a, 3b), baited with test stimuli (4a, 4b = inverted lid with/without cow feces; 5a, 5b = moist/dry paper). The stimulus chambers were connected through a male-female joint (6a, 6b) to interception chambers (7a, 7b) fitted with an internal funnel that allowed responding flies to enter but not exit. The black cloth (8) covering the central dome prompted the flies to remain in the lower part of the dome and to respond to olfactory cues in stimulus chambers. (B) Still-air single-chamber olfactometer with two oviposition sites, each consisting of a 130-mL jar (9) with or without 85 mL of water, a piece of cotton cloth (10; 20 × 8 cm) secured with a rubber band (11) to the jar rim and folded over an inverted lid (12; 2.5 × 1 cm) which did, or did not, contain cow feces. If the cloth was submerged in water, its surface outside the jar was moist. The cotton wick in the water-filled smaller jar (13; 4 × 6 cm) served as a water source for flies. (C) Still-air two-choice olfactometer consisting of a central chamber (14) and two lateral chambers (15a, 15b). Each lateral chamber was fitted with

an oviposition site as described under B. Mesh funnels (16) allowed flies to sense oviposition site cues and to enter, but not exit, lateral chambers. (D) Paired open-cylinder traps deployed in large-scale (room: 225 × 230 × 230 cm) bioassays. A strip of Tanglefoot adhesive (17) on the inner surface of each cylinder helped capture flies that responded to oviposition site cues. Both oviposition sites resembled those described under B, except that each jar contained water with or without ammonium bicarbonate and that the inverted lid was larger (6 × 1 cm) to hold 20 g of fish food.

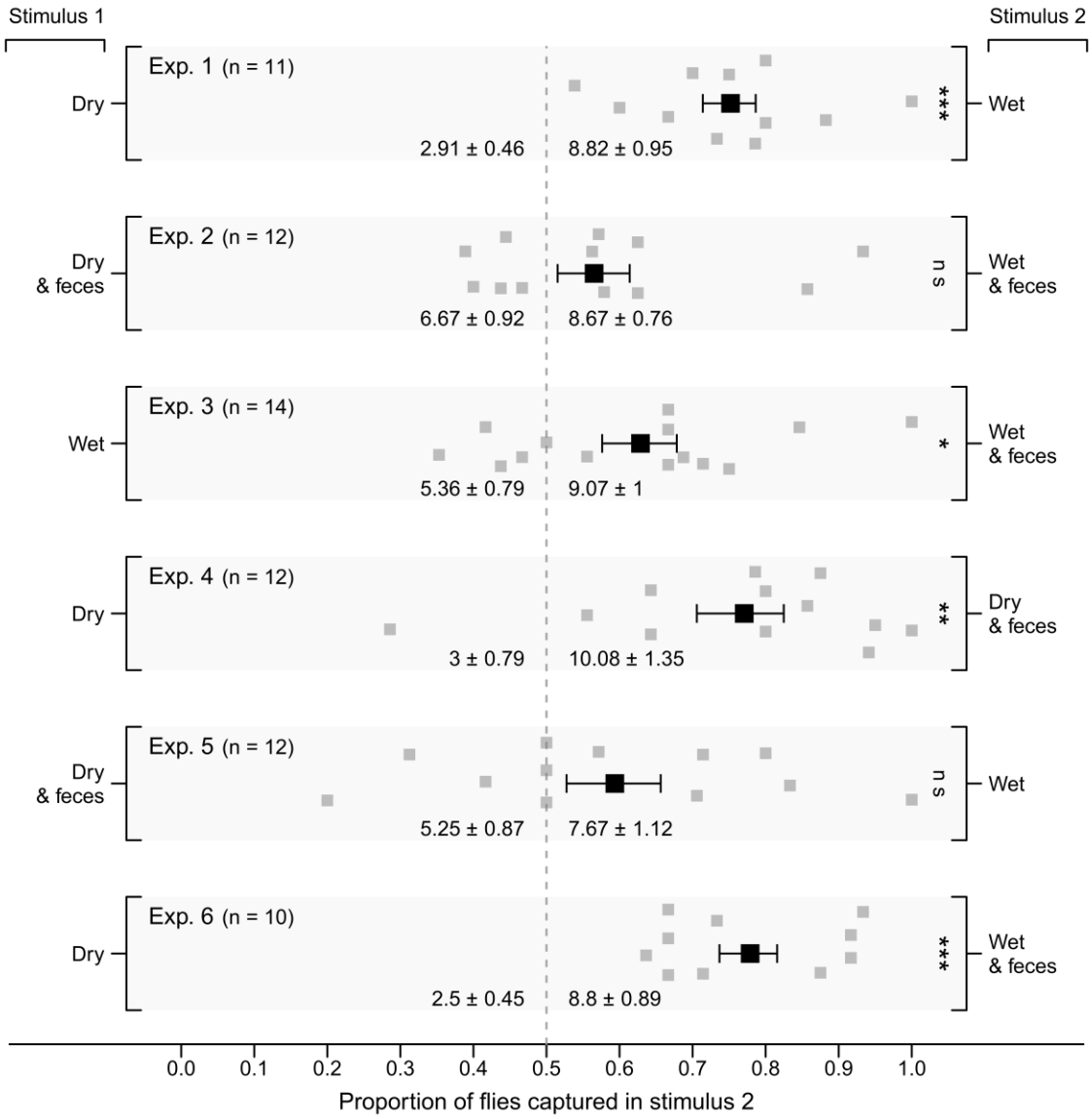

Figure 2.2 Proportions of gravid female stable flies captured in the interception chambers of a moving-air olfactometer (see Figure 2.1A) when they responded to physical and chemical characteristics of prospective oviposition sites (presence or absence of cow fecal smell and/or moisture). For each bioassay replicate, 20 gravid females were released into the central chamber of the olfactometer. Grey symbols show the proportion of flies captured in individual replicates in response to stimulus 2, whereas black symbols show the mean proportion (± SE). The mean (± SE) numbers of flies responding to test stimuli in each experiment are listed near the vertical dashed line. Likelihood ratio test: *$0.01 < P < 0.05$, **$0.001 < P < 0.01$, ***$P < 0.001$; ns, $P > 0.05$ (Nayani et al. 2022).

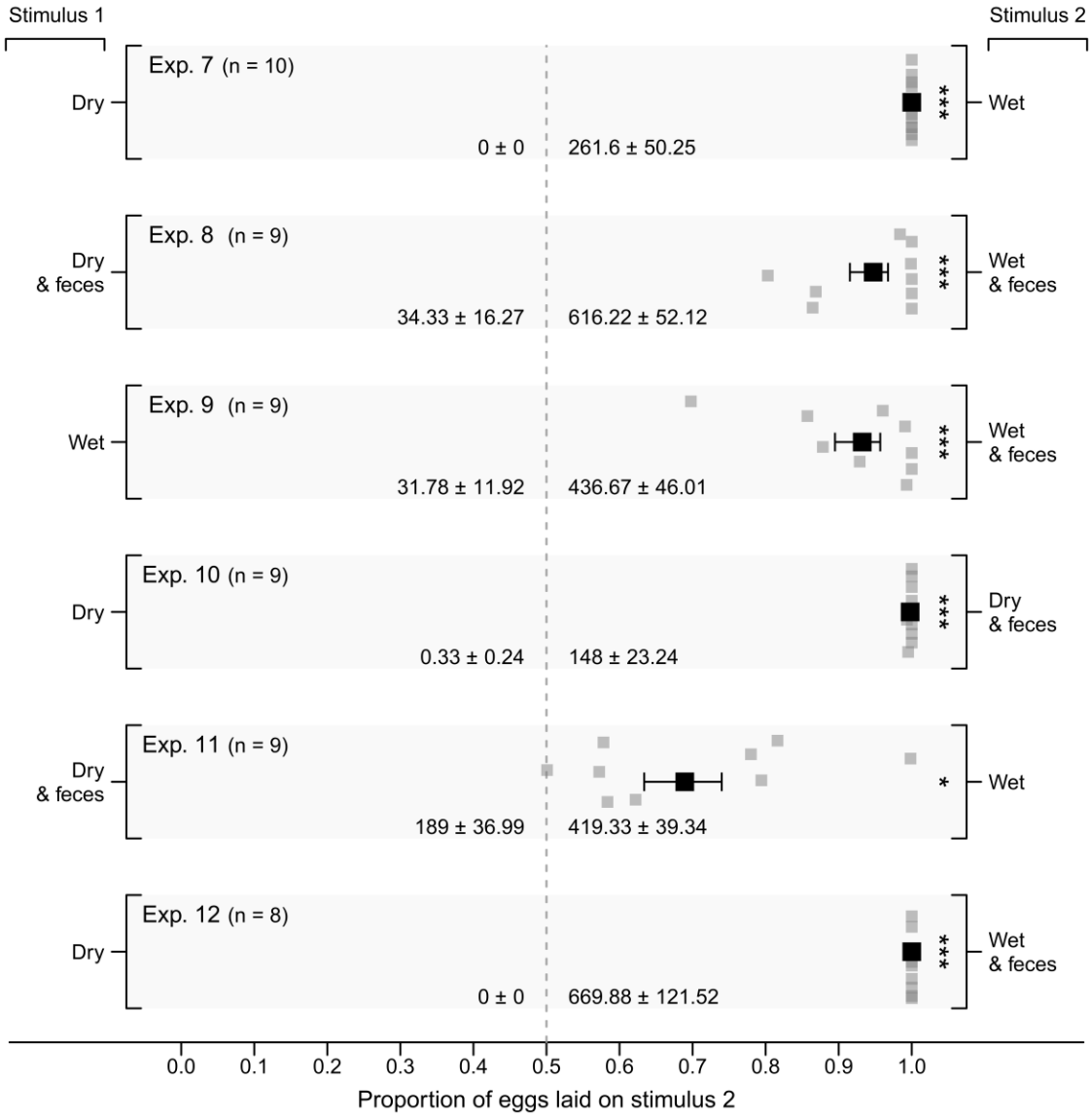

Figure 2.3 Proportions of eggs laid by gravid female stable flies when offered, in still-air single-chamber bioassays, a choice between two oviposition sites (see Figure 2.1B) that differed in physical and chemical characteristics (presence/absence of cow fecal smell and/or moisture). For each experimental replicate, 20 gravid female flies were released into the cage and allowed 24 h to lay eggs. Grey symbols show the proportion of eggs that flies laid in individual replicates in response to stimulus 2, whereas black symbols show the mean proportion (± SE). The mean (± SE) numbers of eggs laid on oviposition sites in each experiment are listed near the vertical dashed line. Likelihood ratio test: *$0.01 < P < 0.05$, ***$P < 0.001$ (Nayani et al. 2022).

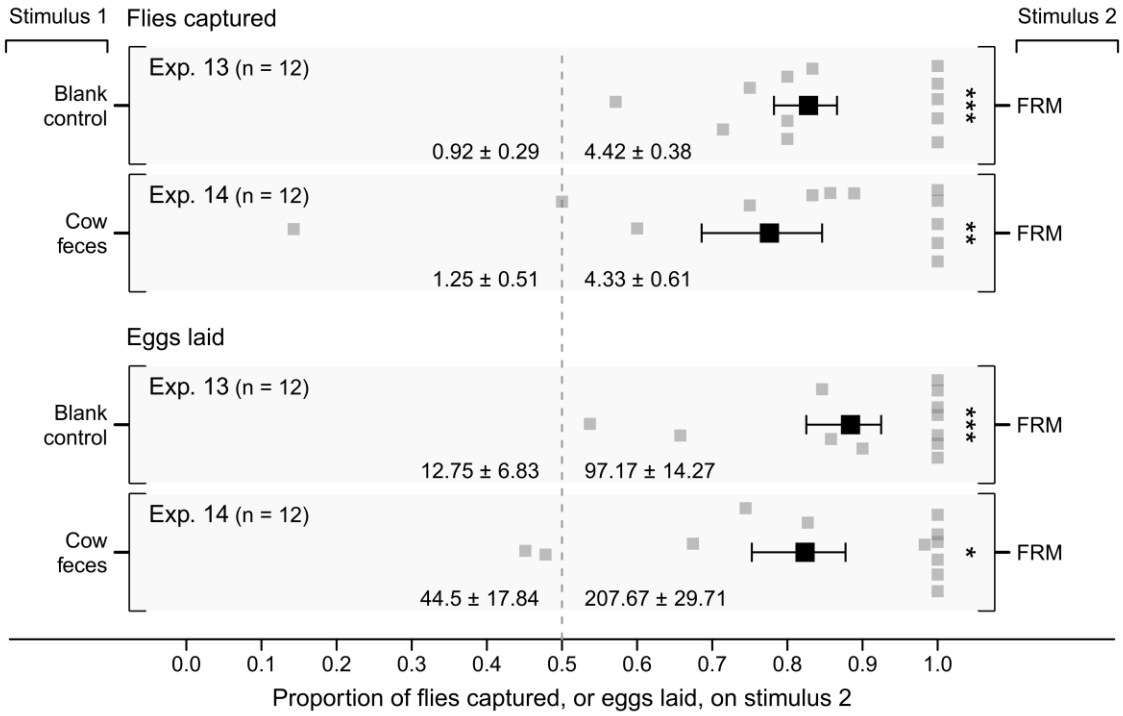

Figure 2.4 Proportion of gravid female stable flies captured, and the eggs they laid, in 3-chamber still-air olfactometer bioassays (see Figure 2.1C) when offered a choice between two oviposition sites that differed in chemical characteristics. For each experimental replicate, 20 gravid female flies were released into the central chamber of the olfactometer and allowed 24 h to approach oviposition sites in lateral chambers and to lay eggs. Grey symbols show the proportion of flies captured, and eggs laid, in individual replicates in response to stimulus 2, whereas black symbols show the mean (± SE). The mean (± SE) numbers of flies captured, and eggs laid, in response to test stimuli in each experiment are listed near the vertical dashed line. Likelihood ratio test: *$0.01 < P < 0.05$, **$0.001 < P < 0.01$, ***$P < 0.001$ (Nayani et al. 2022).

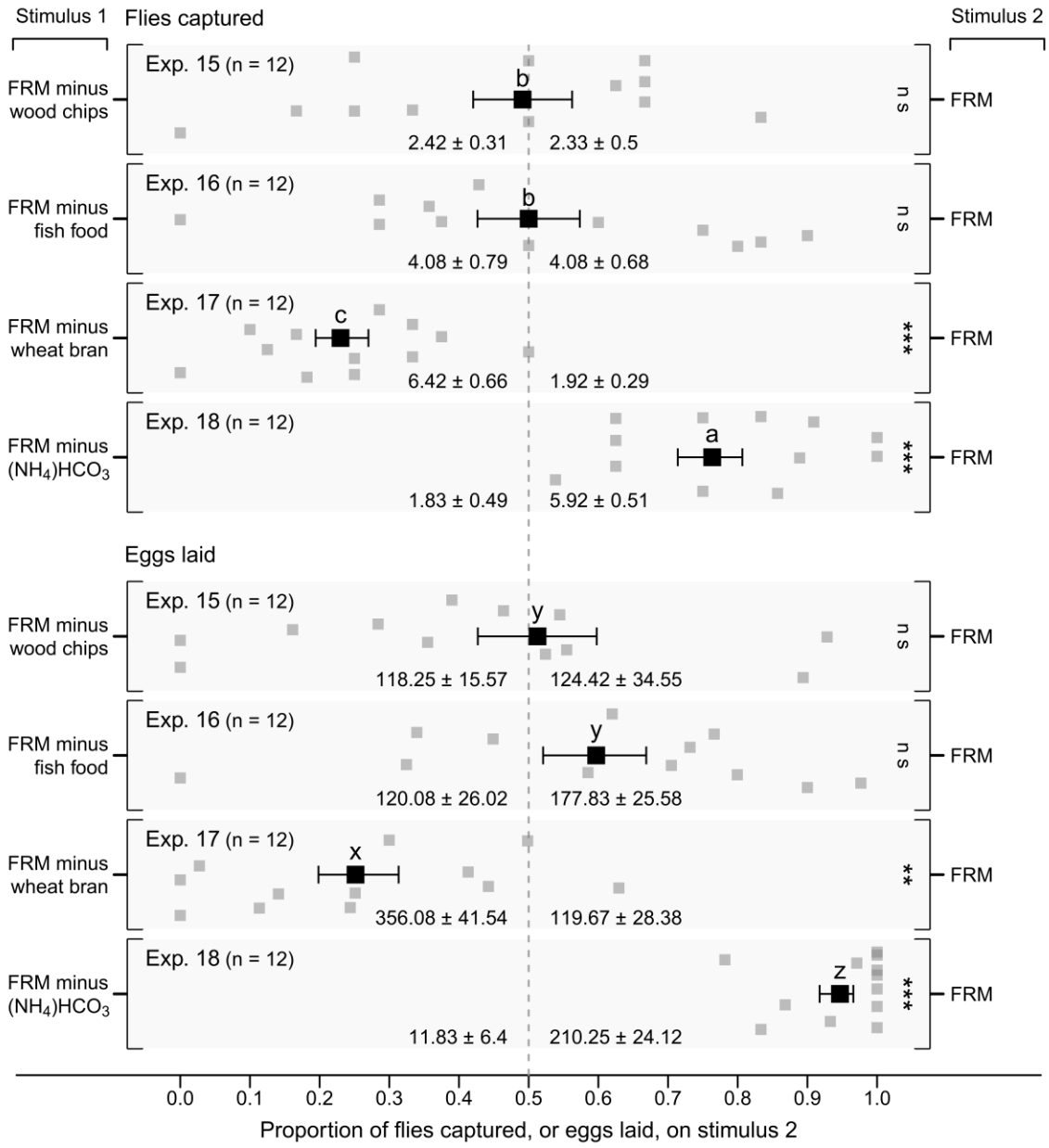

Figure 2.5 Proportion of gravid female stable flies captured, and the eggs they laid, in 3-chamber still-air olfactometer bioassays (see Figure 2.1C) when offered a choice between two oviposition sites that consisted of fly rearing medium (FRM) with four constituents [wood chips, fish food, wheat bran, ammonium bicarbonate $(NH_4)HCO_3$] and medium lacking one of these four constituents. For each experimental replicate, 12 gravid female flies were released into the central chamber of the olfactometer and allowed 24 h to approach oviposition sites in lateral chambers and to lay eggs. Grey symbols show the proportion of flies captured, and eggs laid, in individual replicates in response to stimulus

2, whereas black symbols show the mean proportion (± SE). The mean (± SE) numbers of flies captured, and eggs laid, in response to test stimuli in each experiment are listed near the vertical dashed line. Likelihood ratio test: **0.001 < P < 0.01, ***P < 0.001, ns, P > 0.05 (Nayani et al. 2022). Within each criterion recorded (flies captured and eggs laid, respectively), mean proportions labelled with different letters differ statistically (Tukey test: P < 0.05).

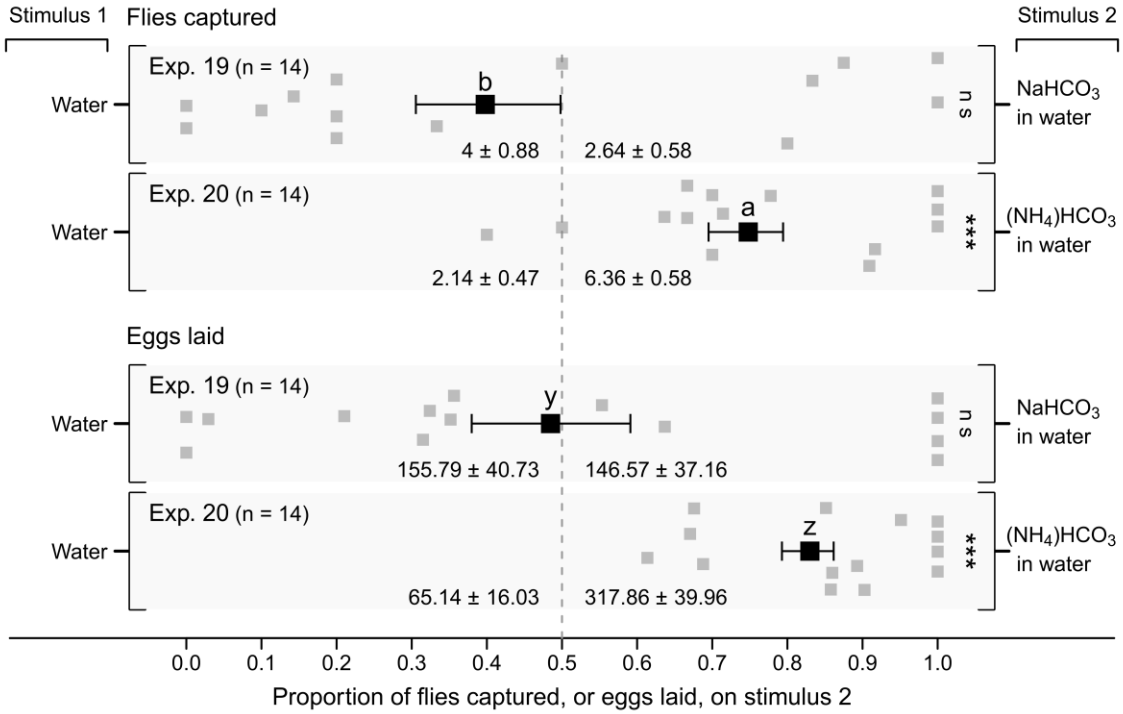

Figure 2.6 Proportion of gravid female stable flies captured, and the eggs they laid, in 3-chamber still-air olfactometer bioassays (see Figure 2.1C) when offered a choice between two moist oviposition sites with or without sodium bicarbonate ($NaHCO_3$, releasing CO_2; Exp. 19) or ammonium bicarbonate [$(NH_4)HCO_3$, releasing NH_3 and CO_2; Exp. 20]. For each experimental replicate, 20 gravid female flies were released into the central chamber of the olfactometer and allowed 24 h to approach oviposition sites in lateral chambers and to lay eggs. Grey symbols show the proportion of flies captured, and eggs laid, in individual replicates in response to stimulus 2, whereas black symbols show the mean proportion (± SE). The mean (± SE) numbers of flies captured and eggs laid in response to test stimuli in each experiment are listed near the vertical dashed line. Likelihood ratio test: ***$P < 0.001$, ns, $P > 0.05$. Within each criterion recorded (flies captured and eggs laid, respectively), mean proportions labelled with different letters differ statistically (Tukey test: $P < 0.05$).

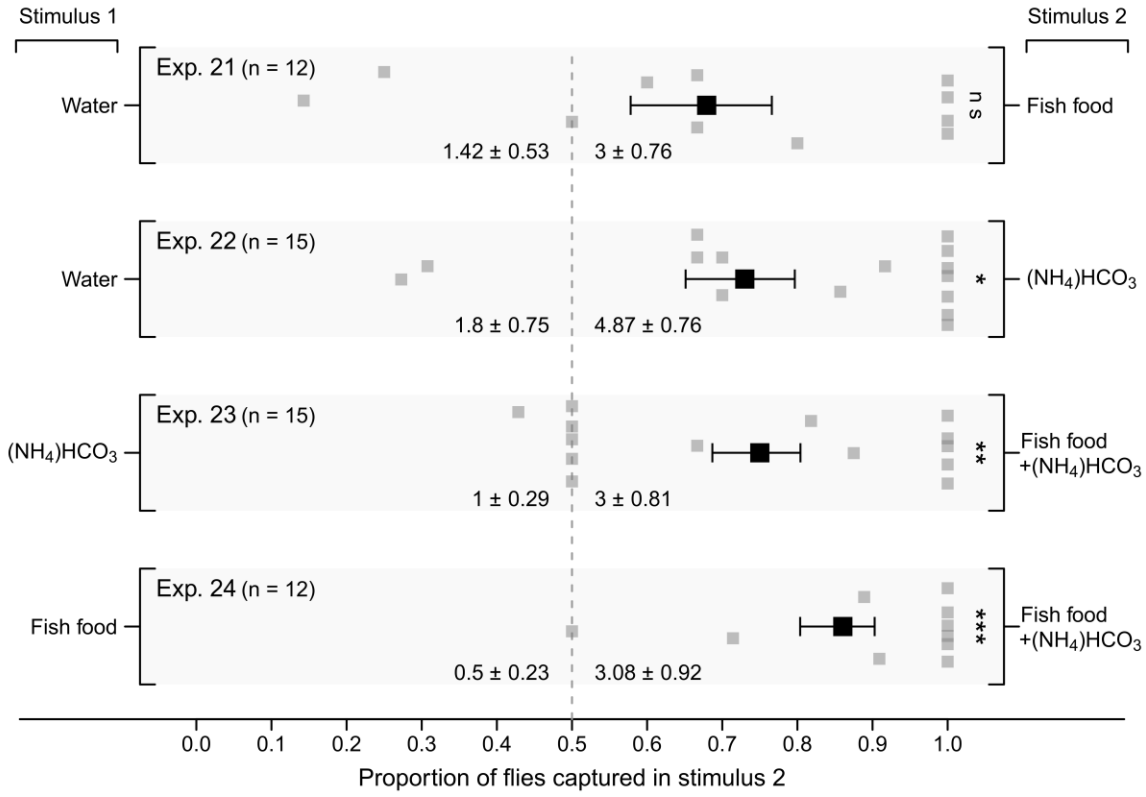

Figure 2.7 Proportion of gravid female stable flies captured in large-scale (room) 2-choice bioassays on adhesive cardboard cylinders enclosing oviposition sites (see Figure 2.1D) that were baited with fish food or water (Exp. 21), $(NH_4)HCO_3$ (releasing NH_3 and CO_2) or water (Exp. 22), fish food + $(NH_4)HCO_3$ or either $(NH_4)HCO_3$ (Exp. 23) or fish food (Exp. 24). For each experimental replicate, 20 gravid female flies were released into the room and allowed 24 h to respond. Grey symbols show proportion of flies captured in individual replicates in response to stimulus 2, whereas black symbols show the mean proportion (± SE). The mean (± SE) numbers of flies captured in response to test stimuli in each experiment are listed near the vertical dashed line. Likelihood ratio test: *$0.01 < P < 0.05$, **$0.001 < P < 0.01$, ***$P < 0.001$; ns, $P > 0.05$.

Table 2.1 Summary of experiments and objectives, number of replicates run, numbers of flies tested per replicate, olfactometers deployed for bioassays (see Figure 2.1), the spatial dimensions (room or wire mesh cage) of non-olfactometer experiments, and the stimuli tested [wet/dry paper towel/cloth, with/without cow feces, fly rearing medium (FRM), or fish food].

Experiment no. (no. replicates)	No. flies/replicate	Olfactometer type/spatial dimensions	Stimulus 1	Stimulus 2
Objective 1: Determine effects of oviposition site moisture and odor on fly attraction and oviposition				
1 (11)	20	Moving-air 2-choice	Dry	Wet
2 (12)	20	Moving-air 2-choice	Dry & feces[1]	Wet & feces
3 (14)	20	Moving-air 2-choice	Wet	Wet & feces
4 (12)	20	Moving-air 2-choice	Dry	Dry & feces
5 (12)	20	Moving-air 2-choice	Dry & feces	Wet
6 (10)	20	Moving-air 2-choice	Dry	Wet & feces
7 (10)	20	Cage[4]	Dry	Wet
8 (9)	20	Cage	Dry & feces	Wet & feces
9 (9)	20	Cage	Wet	Wet & feces
10 (9)	20	Cage	Dry	Dry & feces
11 (9)	20	Cage	Dry & feces	Wet
12 (8)	20	Cage	Dry	Wet & feces
Objective 2: Compare oviposition sites for their ability to attract flies and induce oviposition				
13 (12)	12	Still-air 2-choice	Blank	FRM[1,2]
14 (12)	12	Still-air 2-choice	Feces	FRM
Objective 3: Determine the constituent(s) of a superior oviposition site affecting the flies' attraction and oviposition responses				
15 (12)	12	Still-air 2-choice	FRM	FRM minus wood chips
16 (12)	12	Still-air 2-choice	FRM	FRM minus fish food
17 (12)	12	Still-air 2-choice	FRM	FRM minus wheat bran
18 (12)	12	Still-air 2-choice	FRM	FRM minus $(NH_4)HCO_3$
19 (14)	20	Still-air 2-choice	Water	$NaHCO_3$ in water
20 (14)	20	Still-air 2-choice	Water	$(NH_4)HCO_3$ in water
21 (12)	20	Room[4]	Water	Fish food[1]
22 (12)	20	Room	Water	$(NH_4)HCO_3$ in water[3]
23 (15)	20	Room	$(NH_4)HCO_3$ in water	$(NH_4)HCO_3$ in water[3] & fish food[1]
24 (12)	20	Room	Fish food	$(NH_4)HCO_3$ in water & fish food

[1]Amount of test stimuli: feces, 5 g; FRM, 5 g; fish food, 20 g.
[2]Fly rearing medium contained wood chips, fish food, wheat bran, ammonium bicarbonate (NH_5CO_3), and water as constituents.
[3]3 mL of a 2.4% ammonium bicarbonate [$(NH_4)HCO_3$] solution.
[4]Dimension: room, 225 × 230 × 230 cm; wire mesh cage, 46 × 46 × 46 cm

Table 2.2 Summary of effects of oviposition site characteristics (wet/dry, with/without cow feces) on attraction of stable flies (Exps. 1–6) and their propensity to oviposit (Exps. 7–12), when tested in a full factorial experimental design. Cells where the row stimulus is preferred to the column stimulus are indicated in orange, cells where the reverse is true are indicated in blue. Statistical significance of experiments is indicated by asterisks (*0.01 < P < 0.05, **0.001 < P < 0.01, ***P < 0.001; ns, P > 0.05).

Proportion of flies captured

		No feces		Feces	
		Dry	Wet	Dry	Wet
No feces	Dry		Exp. 1***	Exp. 4**	Exp. 6***
	Wet	Exp. 1***		Exp. 5^{ns}	Exp. 3*
Feces	Dry	Exp. 4**	Exp. 5^{ns}		Exp. 2^{ns}
	Wet	Exp. 6***	Exp. 3*	Exp. 2^{ns}	

Proportion captured, or ovipositing on row stimulus

- 0.0–0.2
- 0.2–0.4
- 0.4–0.6
- 0.6–0.8
- 0.8–1.0

Proportion of eggs laid

		No feces		Feces	
		Dry	Wet	Dry	Wet
No feces	Dry		Exp. 7***	Exp. 10***	Exp. 12***
	Wet	Exp. 7***		Exp. 11*	Exp. 9***
Feces	Dry	Exp. 10***	Exp. 11*		Exp. 8***
	Wet	Exp. 12***	Exp. 9***	Exp. 8***	

Chapter 3: *Staphylococcus* microbes in the bovine skin microbiome attract blood-feeding stable flies

A near identical version of this chapter has been published in *Frontiers in Ecology and Evolution* with the following authors: Saif Nayani, Sanam Meraj, Emerson Mohr, Regine Gries, Emma Kovacs, Anand Devireddy, Gerhard Gries. doi: 10.3389/fevo.2023.1212222

Abstract

The human skin microbiome reportedly contributes to the attraction of mosquitoes to human hosts. We tested the hypothesis that bovine skin microbes affect the attraction of blood-feeding stable flies, *Stomoxys calcitrans,* to their bovine hosts. Microbes were collected from a calf and adult cow, and subsequently isolated and identified by mass spectrometry and genetic sequencing. Separate groups of (*i*) four *Staphylococcus* congeners (*S. chromogenes*, *S. sciuri*, *S. simulans, S. succinus*) and (*ii*) three bacterial heterogeners (*Glutamicibacter protophormiae*, *Corynebacterium stationis*, *Wautersiella* sp.) grown on agar, each attracted flies in still-air olfactometers, as did each *Staphylococcus* congener singly. The four *Staphylococcus* microbes also attracted flies in room bioassays. In greenhouse bioassays with paired black barrels as visual (surrogate host) stimuli, the treatment barrel baited with *S. sciuri* on agar induced significantly more fly alighting responses than the control barrel with sterile agar. This treatment effect could not be demonstrated on a cattle farm, possibly because of chemically and visually complex surroundings. Ammonia emitted by *Staphylococcus* microbes attracted flies, and a synthetic blend of microbe odorants enhanced the attractiveness of ammonia. Optimal attraction of stable flies to bovine microbes likely requires the integration of multimodal host cues.

Keywords: microbe, *Staphylococcus*, *Stomoxys calcitrans*, attraction, semiochemical, ammonia

3.1) Introduction

To locate vertebrate hosts, hematophagous insects exploit multiple host cues (Marzal et al. 2022), including carbon dioxide (CO_2) (Takken 1991; Anderson et al. 2009; Milne et al. 2009; Indacochea et al. 2017), breath volatiles (Warnes and Finlayson 1985), body-derived odor (Ivan Ortiz and Molina 2010), moisture and heat (Cribellier et al. 2020), infrared (IR) radiation (Schmitz et al. 2000), as well as visual cues such as polarized light reflections from dark-coloured fur (Horvath et al. 2017; Meglic et al. 2019). The relative importance of host cues depends on the insect taxon and the spatial scale. For stable flies, *Stomoxys calcitrans,* visual host cues seem particularly important (Murchie et al. 2018; Onju et al. 2020; Sharif et al. 2020; Blake et al. 2023), and are likely sensed over a long range. For mosquitoes, host cues such as body heat, skin odor and moisture are most important at close to intermediate ranges (Marzal et al. 2022). Female mosquitoes respond to host chemical and physical cues in sequential and interactive processes. Exhaled in the breath of a potential host, CO_2 context-specifically elicits host-seeking behavior (Gillies 1980), induces upwind flight toward the CO_2 source (Healy and Copland 1995), and enhances attraction to warmth (Liu and Vosshall 2019). In addition to exhaled CO_2 and breath volatiles, odorants emanating from bacteria on human skin guide host-foraging mosquitoes (Showering et al. 2022).

The common human skin bacteria *Staphylococcus epidermidis*, *Corynebacterium minutissimum*, and *Bacillus subtilis* emit odorants that attract *Anopheles gambiae* mosquitoes (Verhulst et al. 2010). Skin microbiota differ among humans and thus affect their relative attractiveness to mosquitoes (Showering et al. 2022). Humans most attractive to *A. gambiae* have high densities of skin microbes and great abundance of *Staphylococcus* spp., suggesting that *Staphylococcus* spp. contribute to the attractiveness of humans to mosquitoes (Verhulst et al. 2011). As microbes produce species- or strain-specific odor blends (Green et al. 2014; Peach et al. 2021), it follows that the species composition of skin microbiomes also affects its odor profile and thus the attractiveness of humans to host-seeking insects. With the human skin microbiome known to affect mosquito attraction and host recognition (Verhulst et al. 2011), it is conceivable that the skin microbiomes of other vertebrates, such as cattle (Zinicola et al. 2015a), may also affect their attractiveness to blood-feeding insects including stable flies, *Stomoxys*

calcitrans, which are major pests of cattle in livestock production industries. Repeated biting by flies reduces weight gain and milk production (Bruce and Decker 1958; Campbell et al. 1977; Campbell et al. 2001), causing billions of dollars in economic losses per year (Taylor et al. 2012).

Skin microbiota of cattle have been extensively investigated (Winther et al. 2022), particularly within the context of bacterial infections that cause diseases such as mastitis (Andrews et al. 2019; De Buck et al. 2021) and bovine digital dermatitis (Zinicola et al. 2015a,b; Nielsen et al. 2016; Espiritu et al. 2020; Caddey and De Buck 2021; Caddey et al. 2021). Bacteria and their volatile odorant and gas emissions have also been shown to attract stable flies to oviposition sites and to induce oviposition (Romero et al. 2006; Albuquerque and Zurek 2014; Scully et al. 2017). However, whether cattle skin microbes attract stables flies to their cattle hosts has not yet been investigated.

Here, we tested the hypothesis that skin-dwelling microbes of cattle contribute to the attraction of stable flies to cattle hosts. To this end, we had three objectives (O): (O1) identify skin-dwelling microbes of cattle, (O2) test select microbes for their attractiveness to flies, and (O3) investigate mechanisms underlying the attraction of flies to bioactive microbes.

3.2) Material and methods

3.2.1) Rearing of experimental flies

Flies were housed in a hyperbolic growth chamber (BioChambers Inc., Winnipeg, MB, CA) on the Burnaby campus of Simon Fraser University (Nayani et al. 2023a). Briefly, flies were fed citrated bovine blood twice a day and provided cloth oviposition sites at least 3 times per week. Eggs were transferred to a larval rearing medium, containing wood chips (200 g; Hyon Bedding, Prince George, BC, CA), wheat bran (500 g; Rogers Foods, Armstrong, CA), staple fish food (115 g; Nutrafin, Montreal, QC, CA), and a solution of ammonium bicarbonate (50 g) dissolved in 1600–2000 mL of water (Friesen et al. 2018). Reared flies were separated by sex based on morphological and sex-specific characteristics, with males having a visually larger dark spot at the base of their abdomen than females, and females protruding their ovipositor when abdomens are gently squeezed with a pair of forceps. Only female flies, 7–11 days old, were tested in

laboratory bioassays because females – proportionally – responded better than males in pre-screening bioassays.

3.2.2) O1: Identification of microbes on cattle skin

3.2.2.1) Collection and isolation of microbes

Microbes were collected from a live cow calf (with consent and in the presence of the animal's owner), and from the hide of a recently slaughtered adult cow. Samples were obtained from a front leg, back leg, and the back of both the live calf and the cow hide, because these areas are most frequented by stable flies. For microbe collections, cotton swabs (Puritan, Guilford, ME, USA) were dipped in sterile distilled water and then were firmly rubbed for 30 s against an approximately 6.5-cm^2 patch of cow skin/hide before being streaked for 30 s over the entire surface of Mueller Hinton, Yeast Extract, or Potato Dextrose agars in separate Petri dishes (d ≈ 8.5 cm). These different agar types were meant to enable growth of as many bacterial species as possible. One sample was obtained from each body region per agar type, yielding nine samples each from the live calf and the cow hide. Morphologically distinct microbes growing on agar were isolated by continuous re-streaking. All microbial work was done in a biosafety cabinet (BSC; NUAIRE Biological Safety Cabinets, Class II type A2) using aseptic techniques. Microbe stock-samples were kept at -80 °C in a solution of glycerol, distilled water, and liquid microbe culture (1:1:2).

3.2.2.2) Identification of microbes

Isolated microbes were identified using either Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) or genetic sequencing.

MALDI-TOF MS (MALDI-TOF MS; Bruker Corp., Billerica, MA, USA) (Jimenez et al. 2017) was conducted using an extended Direct Transfer method. For each bacterial strain, two preparations were processed. Briefly, after growing unknown bacteria on agar overnight, single colonies were transferred to a well on a MALDI plate via sterile toothpick, producing a heavy smear on the well. The same toothpick was then used to produce a lighter smear on the next well. Subsequently, 1 μL of 70% formic acid was

applied to all microbe-treated wells and allowed to evaporate. Finally, each well received 1 µL of 2-cyano-3-(4-hydroxyphenyl) acrylic acid (HCCA matrix). After wells had dried, MALDI Biotyper measurements were taken. We used a Bruker bacterial test standard (Bruker Corp., Billerica, MA, USA) for calibration in accordance with manufacturer instructions. We analyzed all spectra using Biotyper software (Bruker Corp., Billerica, MA, USA). This Biotyper software calculates an arbitrary score for each sample between 0 and 3 by comparing sample mass spectra to reference mass spectra; we accepted species assignments at scores of >2.0 in accordance with the manufacturer's recommended protocol.

In preparation for genetic sequencing of distinct bacterial colonies, Kodaq PCR Master Mix (Applied Biological Materials, Richmond, CA) was used to amplify the V3-V4 loop of the 16S rRNA gene with the Universal Forward Primer (Uni340F) – 5'-CCTACGGGRBGCASCAG-3' and the Universal Reverse Primer (Uni806R) – 5'-GGACTACNNGGGTATCTAAT-3' (Takai and Horikoshi 2000) by Polymerase Chain Reactions (PCR). Briefly, for a single colony of any unknown bacterium, a PCR mix (25 µL) was prepared using (*i*) 12.5 µL of Kodaq PCR Master Mix, (*ii*) 1 µL of Uni340F, (*iii*) 1 µL of Uni806R, and (*iv*) 10.5 µL of molecular grade water. Both primers were initially at concentrations of 10 µM, resulting in final concentrations of 400 nM of each primer in the final mix. The mix was then deposited in a PCR strip tube and a single colony of the unknown bacterium was added. A PTC-200 Peltier Thermal Cycler (MJ Research, Saint-Bruno-de-Montarville, Quebec, CA) was deployed to run PCR cycles on all samples. The program was set to 94 °C for 2 min, followed by thirty 30-s cycles each at 94 °C, 55 °C, and 72 °C, and a final extension step at 72 °C for 5 min.

The presence of the expected band size at 466 base pairs (bps) and the success of the PCR amplification was checked on a 0.7% agarose gel with a 1 kb Plus Opti DNA Marker (ladder) (Applied Biological Materials, Richmond, BC, CA). PCR amplicons were pooled and concentrated using the QIAquick Gel Extraction Kit and the QIA PCR & Gel Cleanup Kit (Qiagen, Venlo, NL). Amplicons were sequenced (Genewiz, South Plainfield, USA) and the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) was used to compare the sequenced region of individual isolates with known sequences in the National Center for Biotechnology Information's National Library of

Medicine's standard sequence database. A species or genus was determined to be a match, if there was at least 95% coverage and 99% identity between a known sequence and the sequenced isolate.

In total, 38 microbes were identified using MALDI-TOF MS, of which 14 were also identified using genetic sequencing. The sequences of these isolates are provided in the file entitled 'List of microbial species, methods of identification and applicable sequences.xlsx' as part of the supplementary data publication (Nayani et al. 2023b).

3.2.3) O2: Testing of select microbes for their attractiveness to flies

3.2.3.1) Still-air olfactometer bioassays – general experimental design

Bioassays were run in still-air olfactometers, each with a central and two lateral chambers (Figure 3.1A), where treatment and control stimuli were placed. For each experimental replicate, 20 female flies that had been blood- and water-deprived for 24 h were released into the central chamber from which they could enter, but not exit, lateral chambers through mesh funnels. Experimental replicates were terminated and scored after 24 h by placing olfactometers in a freezer (-15°C) and counting the cold-euthanized flies in each chamber.

3.2.3.2) Still-air olfactometer bioassays – specific experiments (Exps. 1–12)

Of the 38 microbes collected from cattle skin and identified to genus and/or species (see Results; Table 3.2), those previously reported on vertebrate skin were tested for their attractiveness to stable flies. These microbes included four *Staphylococcus* congeners [*S. sciuri*, *S. succinus*, *S. simulans*, *S. chromogenes* (referred to as "Group 1")] and three heterogeners [*Glutamicibacter protophormiae*, *Corynebacterium stationis*, *Wautersiella* sp. (referred to as "Group 2")]. For Experiment 1 (n = 12) (Table 3.1), each of the four Group-1 *Staphylococcus* species was grown overnight on agar in a petri dish (d ≈ 8.5 cm), and then one quarter slice of the agar from each species was placed in a new sterile petri dish serving as the treatment stimulus, whereas four slices of sterile agar served as the control stimulus. Agar was sliced, and slices were transferred, using sterile (autoclaved) popsicle sticks. Test stimuli involving Risk Group 2 microbes (Government of Canada) were prepared either near a flame or in a biosafety cabinet. These microbes

included: *S. simulans*, *G. protophormiae*, and *Wautersiella* sp. (as Risk Group classification for the latter two was not available, RG2 safety measures were taken as a precaution). For Experiment 2 (n = 12), the same procedure was applied with the three Group-2 microbes except that only three quarter slices were used for the treatment stimulus and three corresponding sterile slices for the control stimulus.

Drawing on results that treatment stimuli in both experiments 1 and 2 attracted significantly more flies than corresponding control stimuli (Figure 3.2), but that Group-1 microbes seemed more attractive (mean treatment to control response ratios: Group 1: 9.5 to 1; Group 2: 4.4 to 1), follow-up experiments were designed to determine the key microbe(s) in Group 1 that mediated attraction of flies. To this end, parallel experiments 3–7 (n = 10 each) tested all four Group-1 microbes (Exp. 3; positive control), and Group-1 microbes without *S. chromogenes* (Exp. 4), *S. sciuri* (Exp. 5), *S. simulans* (Exp. 6), or *S. succinus* (Exp. 7), all *versus* sterile agar controls. With evidence that the deletion of any one *Staphylococcus* species from Group 1 did not reduce the Group's attractiveness (see Results, Figure 3.3), experiments 8–12 (n = 10 each) then tested the four Group-1 *Staphylococcus* microbes in combination (Exp. 8; positive control) and singly (Exps. 9–12), again all *versus* sterile agar controls.

3.2.3.3) Room bioassay (Exp. 13)

To determine whether Group-1 *Staphylococcus* microbes attract stable flies not only in small scale olfactometers but also over a long range, experiment 13 (n = 20) was set up in a laboratory room (230 × 230 × 225 cm high), with the treatment stimulus (one quarter agar slice of each of the four *Staphylococcus* microbes) and the control stimulus (sterile agar) prepared as described for experiment 1 (see above subsection). The room was kept at a temperature of approximately 26 °C and lit by a combination of plant illumination lights (Standard Products Inc., Saint-Laurent, QC, CA) and day lights (Philips, Amsterdam, NL), set to maintain a photoperiod of 15 h L: 9 h D. The treatment and control plates were then placed on the room floor and surrounded by vertical black cardstock cylinders (9.5 × 28 cm high) (Figure 3.1B), which were coated on the inside with adhesive Tanglefoot® (Tanglefoot, Marysville, USA). For each experimental

replicate, 20 flies were released into the room, and fly captures in the cardboard cylinder traps were recorded 24 h later.

3.2.3.4) Greenhouse bioassay (Exp. 14)

To test whether a Group-1 microbe affects the responses of flies not only in a room setting (see Results; Figure 3.5) but also on an even larger scale, experiment 14 (n = 10) was set up in a greenhouse compartment (600 × 600 × 360 cm high) on the Burnaby campus of Simon Fraser University. *Staphylococcus sciuri* was selected for greenhouse bioassays, and subsequent field bioassays (see below), because it was as attractive as either one, and all four, of the Group-1 microbes (see Figure 4), and because it was the safest microbe (Risk Group 1 microbe; Government of Canada) for deployment in large-scale settings, being deemed unlikely to cause human or animal diseases.

In the greenhouse bioassay, the paired test stimuli consisted of barrels (38 × 64 cm high) covered in black cloth and placed on metal platforms 71 cm above ground, and 200 cm apart from each other (Figure 3.1C). In each replicate, three agar plates were secured with double-sided tape to each platform, with two plates at the front and one plate at the back of the barrels' curved surface. Treatment and control plates were covered in *S. sciuri* (grown overnight) and kept sterile, respectively. To record alighting by flies on barrels and agar plates, video cameras (Akaso, Frederick, MD, USA) were mounted on stands 83 cm above ground and 100 cm away from both the front and the back of each barrel. To initiate a bioassay replicate, the cameras were turned on, and 100 blood- and water-deprived female flies were released into the compartment, 300 cm away from treatment and control stimuli. Ten minutes later, video recordings were stopped and flies were sweep-netted and released outside. Videos were subsequently examined to determine the number of times flies landed on each of the two barrels over the bioassay period.

3.2.3.5) Field bioassays (Exps. 15–18)

With evidence that *S. sciuri* affected alighting responses by flies in the greenhouse compartment (see Results; Figure 3.6), experiments 15–18 (n = 10 each) then tested the effect of *S. sciuri* on fly attraction at a livestock farm (Eagle Acres Dairy; Langley, BC, CA). A near-identical experimental design (Figure 3.1D) as in the greenhouse experiment

was used, except that the platform of each paired barrel was fitted with 12 plates (Exp. 15), 4 plates (Exp. 16), 2 plates (Exp. 17), and 1 plate (Exp. 18). Barrels were placed approximately 300 cm away from the barn in all experiments. Experimental replicates were terminated after 5 min, and positions of treatment and control barrels were alternated between replicates.

3.2.4) O3: Investigation of mechanisms underlying attraction of flies to *Staphylococcus* spp.

3.2.4.1) Evidence for ammonia (NH_3) emission from Staphylococcus microbes

Having shown that ammonia serves as an oviposition resource cue to stable flies (Nayani et al. 2023a), here we tested whether ammonia also functions as a host-foraging cue for flies. To this end, we tested whether *Staphylococcus* microbes collected from cattle skin emit ammonia, and whether ammonia attracts host-foraging flies in bioassays. To test for ammonia emission, we grew microbes on agar overnight and measured ammonia emission, using a MultiRAE Wireless Portable Six-Gas Monitor (Honeywell, Charlotte, NC, USA). Three plates of each microbe were grown for measurement of gas emissions. Measurements were taken by placing the probe of the gas meter 0.5-1.0 cm above an agar plate, waiting 1 min for readings to stabilize, and then recording the ppm of ammonia. Between replicates, the gas meter was kept in regular laboratory air for 1 min, thus allowing readings to return to baseline ammonia levels in the atmosphere.

3.2.4.2) Ammonia bioassays in still-air olfactometers (Exps. 19–21)

With evidence that microbes attractive to flies emit ammonia (see Results; Table 3.3), parallel still-air olfactometer experiments 19–21 (n = 20 each) then tested whether ammonia on its own is attractive to host-foraging flies. For both the treatment and the control stimulus in these experiments, sodium chloride solutions (50 g NaCl in 50 mL water) were prepared, with treatment solutions also containing ammonium bicarbonate (NH_4HCO_3) at 0.1 g (Exp. 19), 1 g (Exp. 20), or 10 g (Exp. 21). Aliquots (3 mL) of treatment solutions were pipetted into inverted bottle caps (2.5 × 1 cm; total volume: 4.9 mL), and ammonia ppm for each NH_4HCO_3 dose (0.1 g, 1.0 g or 10 g in 50 mL water) was measured 0.5 cm above the liquid surface. The mean ammonia ppm measured at 0.1

g, 1.0 g, and 10 g in seven replicates each was 0.42 ppm, 5.6 ppm, and >25 ppm (sensor overload), respectively, well within the ppm range of *Staphylococcus* microbes growing on agar (Table 3.3). Carbon dioxide ppm was not measured.

Aliquots (2.5 mL) of treatment and control solutions were transferred to inverted bottle caps (see above), placed on jars (5.5 × 7 cm; total volume ≈ 166 mL) filled two-thirds with water, and covered with a piece of wet black cloth (Figure 3.1E) (Nayani et al. 2023a; Friesen et al. 2018). After treatment and control jars were randomly assigned to the lateral chambers of olfactometers, experimental replicates were initiated by releasing 20 blood- and water-deprived female flies into the central chamber of olfactometers, allowing them to enter, but not to exit, lateral chambers through mesh funnels. Replicates were terminated 24 h later by counting the number of flies in lateral treatment and control chambers.

3.2.4.3) Collection of microbe-derived headspace volatiles

Headspace volatiles were collected from the four strains of *Staphylococcus* bacteria that elicited significant behavioral responses from flies in olfactometer experiments. To this end, 10 agar plates were plated with a microbe of interest and incubated overnight. These plates, with open lids, were then placed into a glass chamber (diameter = 19 cm, height = 29.5 cm) connected to a vacuum pump (Neptune Dyna-pump). Charcoal-filtered air was drawn at a flow rate of 1 L · min^{-1} for 24 h through the chamber and subsequently through a glass column (6 mm outer diameter × 150 mm) containing 200 mg of manufacturer-preconditioned Porapak-Q™ adsorbent (50–80 mesh; Waters Associates, Milford, MA, USA). Volatiles were desorbed from Porapak-Q with one rinse of pentane and ether (1:1; 2 mL), and volatile extracts were concentrated to 0.5 mL and kept at 4 °C prior to analyses. All glassware was cleaned with Sparkleen (Thermo Fisher Scientific, MA, U.S.A), rinsed with distilled water, and oven-dried at 130 °C prior to starting a new aeration.

3.2.4.4) Analyses of microbe headspace volatiles by GC-MS

Aliquots of Porapak-Q headspace volatile extracts were analyzed by gas chromatography-mass spectroscopy (GC-MS), using an Agilent 5977 Series 96MDS

coupled to an Agilent 7890B GC (Agilent Technologies Inc., Santa Clara, CA, USA). The instrument was operated in full-scan electron ionization mode and fitted with a DB-5 GC-MS column (30 m × 0.25 mm ID, film thickness 0.25 μm; Agilent Technologies). The injector port, MS source, and MS quadrupole were set to 250, 230, and 150 °C, respectively. Helium was used as a carrier gas (35 cm s^{-1}; 5:1 split ratio), with the following temperature program: 40 °C (held 5 min), 10 °C · min^{-1} to 280 °C (held 10 min). Compounds were identified by comparing their mass spectra and retention indices (relative to aliphatic alkanes (van Den Dool and Dec. Kratz 1963)) with those of authentic standards that were purchased or synthesized in our laboratory (Table 3.3). Each compound was quantified by comparing its area count with that of an external standard run at 1, 10 and 100 ng/μL.

3.2.4.5) Behavioural experiments with synthetic microbe headspace volatiles (Exps. 22–25)

With evidence that microbe-derived ammonia attracts flies (see Results; Figure 3.8), and that microbes also emit complex volatile blends (see Results, Table 3.3), experiments 22–25 were designed to test whether blends of synthetic microbial volatiles enhance the attractiveness of ammonia. Blend 1 consisted of all constituents common in the headspace of the four bioactive *Staphylococcus* species (Table 3.3, Table 3.4), whereas blend 2 consisted of all volatiles emitted by *S. sciuri* (Table 3.3, Table 3.4). Blends 1 and 2 were formulated separately in mineral oil to achieve sustained release of volatiles during the 24-h experimental period. Sustained release was tracked through capture and analyses of headspace volatiles from mineral oil formulations (as described in the previous two subsections). Formulations were adjusted until their headspace blends matched those produced by bacteria. Both treatment and control stimuli consisted of ammonium bicarbonate solutions prepared and presented as in experiment 19 described above, whereas treatment stimuli also presented blend 1 or 2 in mineral oil (Table 3.3, Table 3.4), with plain mineral oil being the corresponding control stimulus.

To address potential effects of 3-dimensional scale on responses of flies (see Nayani et al. 2023a), experiments 22–24 (n = 12 each) were run in still-air olfactometers (Figure 3.1E), and experiment 25 (n = 10) was run in bioassay rooms (230 cm × 230 cm × 225

cm high) (Figure 3.1F), using experimental designs and protocols described above. As the bioactivity of synthetic volatile blends can be dose-dependent (Nyasembe et al. 2012; Wondwosen et al. 2021), we tested the synthetic '*Staphylococcus* blend' (Table 3.4) formulated in mineral oil at three doses in parallel experiments 22–24: the dose described in Table 3.4 (Exp. 22), diluted 10× (Exp. 23), and diluted 100× (Exp. 24). By the time we tested the synthetic '*S. sciuri* blend' (Table 3.4) in experiment 25, we did not know the results of experiments 22–24 (see Results; Figure 3.9), which would have prompted us to test the '*S. sciuri* blend' at a lower dose. However, re-running the *S. sciuri* blend at a lower dose was not possible due to logistic constraints.

3.2.5) Statistical analyses

Data of all experiments were analyzed with binomial generalized linear models (BGLMs), using quasibinomial errors to account for overdispersion (RStudio v4.1.1) (Crawley 2007; Nayani et al. 2023a,b; RStudio Team 2023). These analyses compared an intercept-only model to a null model with a likelihood ratio test to determine whether the proportions of flies responding to treatment stimuli differed from a hypothetical 0.5 proportion. To test for differences in proportions among experiments sharing a common stimulus (Experiments 3–7, 8–12, and 19–21), similar generalized linear models with data from multiple experiments were created. Models with an individual intercept for each experiment were compared to a model with a single intercept, again with a likelihood ratio test. When a significant difference between experiments was observed, the data of these experiments were compared using a post-hoc Tukey test for honestly significant differences in proportions of flies attracted to various treatment stimuli (Hothorn et al. 2008; Nayani et al. 2023b). A p-value of < 0.05 was considered significant in all experiments. Details of all statistical analyses are reported in 'Summary of statistics.xlsx' (Nayani et al. 2023b).

3.3) Results

3.3.1) O1: Identification of microbes collected from cattle skin/hide

Thirty-eight microbial species in 22 genera were isolated and identified (Table 3.2). These genera included *Acinetobacter*, *Alcaligenes*, *Bacillus*, *Burkholderia*, *Candida*,

Citrobacter, *Corynebacterium*, *Enterobacter*, *Escherichia*, *Glutamicibacter*, *Klebsiella*, *Kurthia*, *Lampropedia*, *Pantoea*, *Proteus*, *Pseudochrobactrum*, *Pseudomonas*, *Serratia*, *Staphylococcus*, *Stenotrophomonas*, *Wautersiella*, and *Wickerhamomyces*.

3.3.2) O2: Attractiveness of select microbes to flies

3.3.2.1) Still-air olfactometer bioassays (Exps. 1–12)

Proportionally, more stable flies were attracted to *Staphylococcus* Group-1 bacteria and to heterogeneric Group-2 bacteria than to corresponding sterile agar controls (Figure 3.2, Exps. 1, 2; $p < 0.05$ each). As Group-1 microbes seemed more attractive to flies than Group-2 microbes (mean treatment to control response ratio of flies: Group 1: 9.5 to 1; Group 2: 4.4 to 1), all follow-up experiments were designed to determine the key microbe(s) in Group 1 that mediated fly attraction.

In parallel experiments 3–7 (Figure 3.3), the four Group-1 microbes in combination (positive control) attracted, proportionally, more flies than sterile agar controls (Exp. 3; $p < 0.05$), as did Group-1 microbes without *S. chromogenes* (Exp. 4; $p < 0.05$), *S. sciuri* (Exp. 5; $p < 0.05$), *S. simulans* (Exp. 6; $p < 0.05$), or *S. succinus* (Exp. 7; $p < 0.05$). In parallel experiments 8–12 (Figure 3.4), the four Group-1 microbes in combination attracted, proportionally, more flies than sterile agar controls (Exp. 8; $p < 0.05$), as did each of the four microbes singly (Exps. 9–12; $p < 0.05$ each).

All data combined indicate that single *Staphylococcus* species attract stable flies as effectively as all four *Staphylococcus* species in combination.

3.3.2.2) Room bioassay (Exp. 13)

In a large laboratory bioassay room, adhesive-coated cardboard cylinder traps baited with the four Group-1 *Staphylococcus* microbes on agar captured, proportionally, more flies than cylinder traps baited with sterile agar controls (Figure 3.5, Exp. 13; $p < 0.05$), indicating that microbes affected the responses of flies also in a large bioassay room setting.

3.3.2.3) Greenhouse bioassay (Exp. 14)

In a large greenhouse compartment, black barrels baited with *S. sciuri* on three agar plates prompted, proportionally, more alighting responses by flies than the black barrels baited with three sterile agar control plates (Figure 3.6, Exp. 14; $p < 0.05$), indicating that *S. sciuri* as a single microbe species modulated the responses of flies in a large-scale setting.

3.3.2.4) Field bioassays (Exps. 15–18)

On a cattle farm, black barrels baited with *S. sciuri* on twelve agar plates (Exp. 15), four plates (Exp. 16), two plates (Exp. 17), and on one plate (Exp. 18), all did not prompt proportionally more alighting responses by flies than black barrels baited with the corresponding number of sterile agar control plates (Figure 3.7; $p > 0.05$ for all experiments).

3.3.3) O3: Mechanisms underlying fly attraction to *Staphylococcus* spp.

3.3.3.1) Ammonia emission from Staphylococcus *microbes*

Each of the four Group-1 *Staphylococcus* species emitted ammonia, as follows: *S. chromogenes*: 8.0 ± 1.6 ppm; *S. sciuri*: 14.3 ± 2.6 ppm; *S. simulans*: 13.0 ± 0.0 ppm; *S. succinus:* 9.3 ± 0.9 ppm (Table 3.3).

3.3.3.2) Effect of ammonia on fly attraction (Exps. 19–21)

In still-air olfactometers (Figure 3.1E), all test stimuli containing ammonium bicarbonate, and thus emitting ammonia, attracted proportionately more flies than control stimuli lacking ammonium bicarbonate (Figure 3.8; Exps. 19–21; $p < 0.05$ each). The amount of ammonium bicarbonate in test stimuli did not affect the proportional response level of flies ($p > 0.05$), suggesting that the emission of ammonia, rather than its concentration, affected behavioural responses of flies, at least in this experimental context.

3.3.3.3) Analyses of microbe headspace volatiles by GC-MS

Each of the four Group-1 *Staphylococcus* species emitted a complex volatile blend (Table 3.3). Volatiles shared by all four species included two alcohols (isoamyl alcohol,

phenylethyl alcohol), four pyrazines (2,5-dimethyl pyrazine, trimethyl pyrazine, 2-isopropyl-5-methyl-pyrazine, 2-ethyl-3,5(6)-dimethylpyrazine [composed of 50% 2-ethyl-3,5-dimethylpyrazine and 50% 2-ethyl-3,6-dimethylpyrazine]), and isoamyl acetate (Table 3.3).

3.3.3.4) Effect of synthetic microbe headspace volatiles on fly attraction (Exps. 22–25)
The 'synthetic *Staphylococcus* blend' (Table 3.4) – at a 100× dilution – enhanced attraction of flies to ammonia (Figure 3.9; Exp. 24, $p < 0.05$), but the blend was not effective at a 10× dilution or without dilution (Exps. 22–23, $p > 0.05$ each), with either of these higher-dose blends being less attractive than the 100× diluted synthetic *Staphylococcus* blend (Figure 3.9).

The 'synthetic *S. sciuri* blend' (Table 3.4) did not enhance attraction of flies to ammonia (mean ± SE number of flies responding to treatment and control stimuli: 1.6 ± 0.56 *vs* 1.0 ± 0.49; Exp. 25, $p > 0.05$), but in retrospect should also have been tested at lower doses (see above).

3.4) Discussion

Our data support three conclusions: (1) the cattle skin microbiome is diverse; (2) *Staphylococcus* spp. as members of the cattle skin microbiome are attractive to stable flies; and (3) attraction of stable flies to *Staphylococcus* microbes is mediated by microbe-derived gases and odorants.

To test the hypothesis that skin-dwelling microbes of cattle contribute to the attraction of stable flies to cattle hosts, we could – logistically – bioassay only some of the 38 microbes that we had isolated from cattle skin and identified to the genus and/or species level (Table 3.2). To narrow down the list of the most promising candidate microbes for testing, we focused on those four microbe genera that had previously been reported to be present on animal skin: *Corynebacterium, Glutamicibacter, Wautersiella,* and *Staphylococcus*. As evident from the literature, there are many skin-dwelling or skin commensal microbes in the genera *Corynebacterium* (Corynebacteriaceae) (Cogen et al. 2008; Kong and Segre 2012; Oh et al. 2012; Belkaid and Segre 2014; Ross et al. 2017; Byrd et al. 2018), *Glutamicibacter* (Micrococcaceae) (Noble 1969; Holland et al. 1977,

1979; Rennie et al. 1991; Bernadsky and Rosenberg 1992; Ashbee et al. 1993; Bojar et al. 1995; Harvey and Lloyd 1995; Messiaen et al. 2019), *Wautersiella* (Weeksellaceae) (Ross V et al. 2019; Boxberger et al. 2020; Ma et al. 2021; Wang et al. 2021), and *Staphylococcus* (Staphylococcaceae) (Verhulst et al. 2011; Oh et al. 2014; Ahle et al. 2020). To further streamline behavioural procedures, we established two microbe bioassay groups. We assigned the four identified *Staphylococcus* congeners (*S. sciuri*, *S. succinus*, *S. simulans*, *S. chromogenes)* to Group 1, and the three identified heterogeners (*Glutamicibacter protophormiae*, *Corynebacterium stationis*, and *Wautersiella* sp.) to Group 2. As expected, each group was attractive to stable flies (Figure 3.2), but Group-1 *Staphylococcus* microbes seemed comparatively more attractive (mean treatment to control response ratio by flies: Group 1: 9.5 to 1; Group 2: 4.4 to 1), prompting us to focus on Group-1 microbes in follow-up experiments.

To determine the key microbe(s) in Group 1 that mediated attraction of flies, we tested the Group-1 microbes in their quaternary and all possible ternary combinations, all *versus* sterile agar controls. As the deletion of any one *Staphylococcus* species from Group 1 did not reduce the group's attractiveness (Figure 3.3), we proceeded to test each of the four Group-1 *Staphylococcus* microbes singly. Our findings that each of *S. chromogenes, S. sciuri*, *S. simulans*, and *S. succinus*, on their own attracted stable flies as effectively as all four species combined (Figure 3.4), suggested significant overlap in their headspace volatile blends and gas emissions. Volatile and gas analyses then indeed revealed that isoamyl alcohol, isoamyl acetate, 2,5-dimethyl pyrazine, trimethyl pyrazine, 2-isopropyl-5-methyl-pyrazine, 2-ethyl-3,5(6)-dimethylpyrazine, and phenylethyl alcohol were all common volatiles in the headspace of these *Staphylococcus* congeners, and that each of the four species emitted considerable amounts of ammonia (Table 3.3).

Staphylococcus bacteria are already known to be attractive to dipterans. Humans with a skin flora rich in *Staphylococcus* bacteria are more attractive to African malaria mosquitoes, *Anopheles gambiae,* than humans with a skin flora poor in *Staphylococcus* bacteria or with a greater skin bacterial diversity (Verhulst et al. 2011). Interestingly, the attractiveness of bacteria to mosquitoes is dependent upon the bacterial growth phase. *Staphylococcus epidermidis* in its exponential growth phase (when the rate of increase in bacterial cell numbers is greater than the death rate) was not attractive to *A. gambiae* but

became attractive in its stationary growth phase (when the growth rate is equal to the death rate) (Verhulst et al. 2010), suggesting that the concentration or relative composition of bacterial odor and gas profiles affects foraging decisions by host-seeking mosquitoes. In combination, the data indicate that *Staphylococcus* bacteria contribute to the attractiveness of vertebrate hosts to blood-feeding mosquitoes. A *Staphylococcus* species has also been shown to attract Mexican fruit flies, *Anastrepha ludens*, as do several chemicals in the headspace of *S. aureus* cultures (Robacker et al. 1991, 1993; Robacker and Flath 1995). Finally, *Staphylococcus* bacteria, particularly *S. aureus*, have been implicated in causing bovine mastitis (Taponen and Pyorala 2009). It would be of interest to investigate whether *S. aureus* attracts stable flies and whether stable flies play a role in vectoring *S. aureus* between bovine hosts.

Staphylococcus microbes were attractive to stable flies at three separate scales: a small-scale 3-chamber olfactometer (46 × 21.5 × 15.5 cm) (Figure 3.2; Exp. 1, Figure 3.3; Exp. 3; Figure 3.4; Exp. 8), a medium-scale bioassay room (225 × 230 × 230 cm) (Figure 3.5; Exp. 13), and a large-scale greenhouse compartment (600 × 600 × 360 cm) (Figure 3.6; Exp. 14). Combined, these data suggest that host-foraging stable flies may follow a concentration gradient of microbe-emitted volatile odorants and gases. Similarly, gravid female stable flies responded to volatile odorants and gases (ammonia and carbon dioxide) emanating from prospective oviposition sites, with odorants and gases in combination being most attractive to gravid female flies (Nayani et al. 2023a). In light of all these positive bioassay data, it was perplexing that *S. sciuri,* as a representative of the *Staphylococcus* group, failed to enhance attraction of stable flies to visual targets in field experiments (Figure 3.7). Irrespective of the *S. sciuri* dose (1, 2, 4 or 12 microbe-inoculated agar plates) that was tested, the visual target baited with *S. sciuri* was no more attractive to flies than the paired unbaited control target (Figure 3.7). There are multiple potential explanations for the failure of *S. sciuri* to attract flies in the chemically and visually 'noisy' field setting, as follows: (1) any of the microbe doses tested may still have been suboptimal for fly attraction; (2) growing on agar, *S. sciuri* may have produced an odor and gas profile different from what it typically produces on cattle skin; (3) the odor and gas profile of *S. sciuri* as a single microbe species may have inadequately represented the odor and gas profile of the entire cattle microbiome; (4) in the presence of

complex foraging cues originating from nearby live cattle, a more complex odor profile may have been needed, possibly including odorants and gases emanating not only from the microbiome of cattle but also from their exhale and anus; and (5) for *S. sciuri* to be competitively attractive to flies, further integration of multimodal host foraging cues may be necessary, including visual (Schofield 1998; Cilek 2002; Zhu et al. 2016; Murchie et al. 2018), semiochemical (Jeanbourquin and Guerin 2007a,b; Baleba et al. 2019), thermal, infrared, and aural host foraging cues.

The mechanisms underling attraction of stable flies to *Staphylococcus* microbes involves microbe-produced gases and odorants. All four *Staphylococcus* species identified in our study emitted ammonia (Table 3.3), and ammonia and carbon dioxide emanating from a watery dilution of ammonium bicarbonate attracted stable flies irrespective of the dose tested (Figure 3.8), indicating that ammonia and/or carbon dioxide contribute to the attraction of flies. We predicted that synthetic blends of microbe-derived odorants would also attract stable flies, or would enhance the attractiveness of microbe-produced gases. This prediction was inspired by reports that synthetic odorants attracted tsetse (Saini 1990; Vale 1991), horse flies (Mihok and Lange 2012; Baldacchino et al. 2014), house flies (Cosse and Baker 1996), fruit flies (Robacker et al. 2000; Hanssen et al. 2019), blow flies (Chaudhury et al. 2015; Brodie et al. 2016), and stable flies (Cilek 1999; Jeanbourquin and Guerin, 2007a,b; Mihok et al. 2007; Tangtrakulwanich et al. 2015; Serra et al. 2017). In our study, a synthetic blend of odorants shared between the four *Staphylococcus* species (Table 3.3) enhanced the attractiveness of ammonia and carbon dioxide, revealing an interaction between microbe-produced gases and odorants for fly attraction. Similarly, CO_2 and odorants from deer-associated microbes synergistically attracted Western black-legged ticks, *Ixodes pacificus* (Long et al. 2023). It is remarkable, however, that the bioactivity of odorant blends on attraction of dipterans is contingent upon blend dose (Nyasembe et al. 2012; Wondwosen et al. 2018; this study). In our study, only the 100× dilution of the 'synthetic *Staphylococcus* blend' (Table 3.4) was attractive to stable flies, and synthetic plant volatile blends at low doses were most attractive to *Anopheles* mosquitoes (Nyasembe et al. 2012; Wondwosen et al. 2018). Based on these results there is incentive to re-test the 'synthetic *S. sciuri* blend' (Table 3.4) at a dose lower than previously tested (Figure 3.9).

In conclusion, *Staphylococcus* microbes in the cattle skin microbiome attract stable flies in a manner similar to *Staphylococcus* microbes in the human skin microbiome attracting Malaria mosquitoes. The mechanisms underlying stable fly attraction to cattle skin *Staphylococcus* microbes entail both microbe-derived odorants and gases such as ammonia and/or carbon dioxide. The effect of microbes on fly attraction may be augmented when presented with other cues of the cattle host 'Gestalt'.

3.5) Acknowledgements

We thank Stephen Takács for graphical illustrations in Figure 3.1, Emmanuel Hung for help with the design of surrogate host objects in greenhouse and field experiments, and Adam Blake for help with statistical analyses. We also thank Erin and Brian Anderson as well as their family for allowing us to perform field studies at their farm Eagle Acres Dairy. SN was supported by the Thelma Finlayson Graduate Entrance Scholarship, the Mutual Fire Insurance Company of British Columbia Graduate Scholarship, the Dr. John Yorston Memorial Graduate Scholarship in Biological Sciences and by a Simon Fraser University Graduate Fellowship. Further funding for this project was provided by a Natural Sciences and Engineering Council of Canada (NSERC) – Industrial Research Chair to GG, with BASF Canada Inc., and Scotts Canada Ltd. as the industrial sponsors.

3.6) Data availability

Data, code and applicable sequencing results are available from Mendeley Data: https://doi.org/10.17632/rwdt4dbpzm.1(Nayani et al, 2023b)

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3.8) Figures and Tables

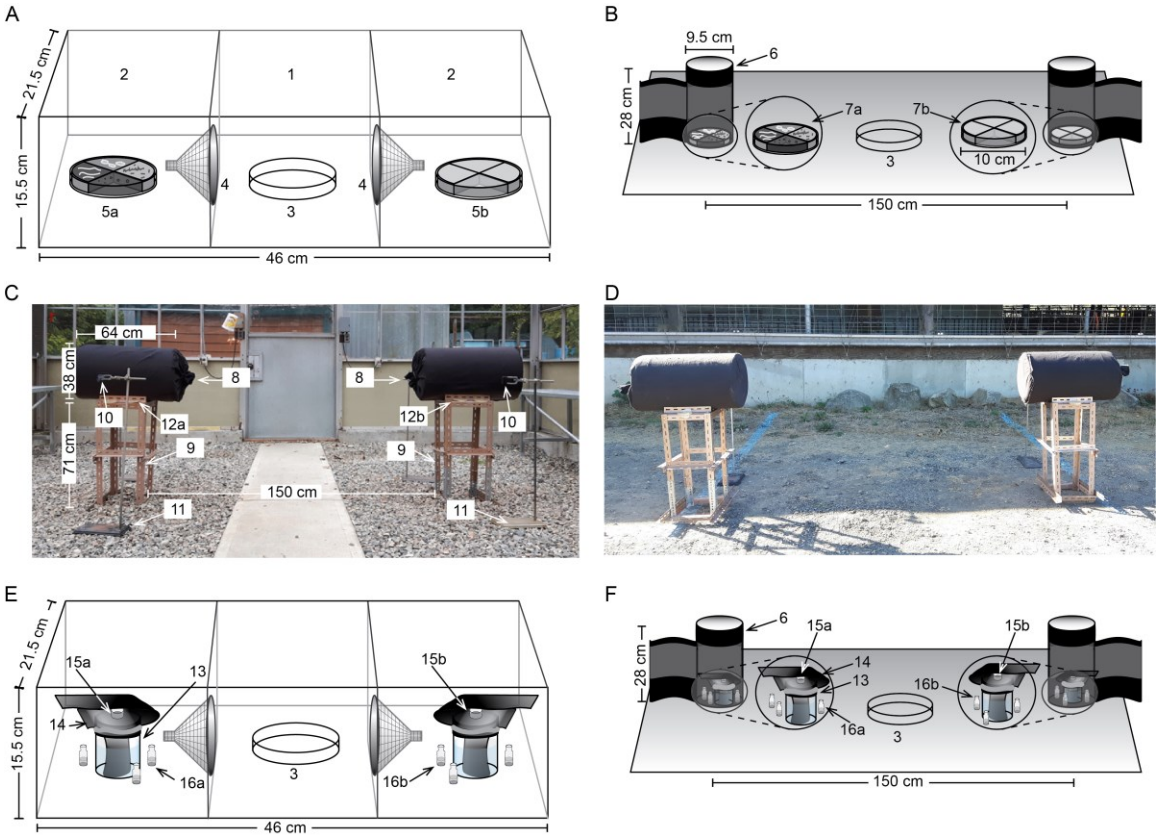

Figure 3.1 Graphic and photographic illustrations of experimental designs. (A) Three-chamber still-air olfactometer with one central (1) and two lateral chambers (2). Flies were released from a petri dish (3) and entered lateral chambers via mesh funnels (4) in response to treatment or control stimuli (5a, 5b). Treatment stimuli consisted of agar slices inoculated, or not (control), with various bacteria (Exps. 1–12; Table 3.1). (B) Trap design and placement in a bioassay room (225 × 230 × 230 cm). Flies were released from a petri dish (3) and captured on cardstock cylinders with an adhesive-coated inner surface (6). The treatment stimulus (7a) consisted of four agar slices each inoculated with *Staphylococcus chromogenes*, *S. sciuri*, *S. simulans* or *S. succinus*, whereas the control stimulus (7b) consisted of corresponding sterile agar slices (Exp. 13). (C) Experimental design employed in a greenhouse compartment (600 × 600 × 360 cm). Alightings of flies on paired black barrels (8) residing on metal stands (9) were recorded by four cameras (10; two shown) mounted on laboratory stands (11). Barrels were baited with three agar

plates inoculated, or not (control), with *S. sciuri* (12a, 12b) (Exp. 14), with two and one plate, respectively, secured at the barrels' curved front and back sides. (D) Experimental design in a field setting. Treatment stimuli consisted of agar plates (12, 4, 2 or 1) inoculated, or not (control), with *S. sciuri* (Exps. 15–18). (E) Three-chambered still-air olfactometer, with test stimuli consisting of a jar filled three-quarters with water (13) in which a black cloth (14) was submerged, secured with a rubber band, and wrapped around the jar top carrying an inverted bottle cap (15a, 15b) filled with a treatment or a control stimulus (Exps. 19–21; Table 3.1). In Experiment 22, both bottle caps (15a, 15b) contained the same stimulus (see Table 3.1), and both lateral chambers were fitted with three vials containing mineral oil, with treatment vials (16a), but not control vials (16b), releasing a synthetic *Staphylococcus* volatile blend (Table 3.4). (F) Trap design and placement as in subpanel B with test stimuli similar to those in subpanel E except that a synthetic *S. sciuri* volatile blend was tested (Table 3.1, Exp. 23; Table 3.4).

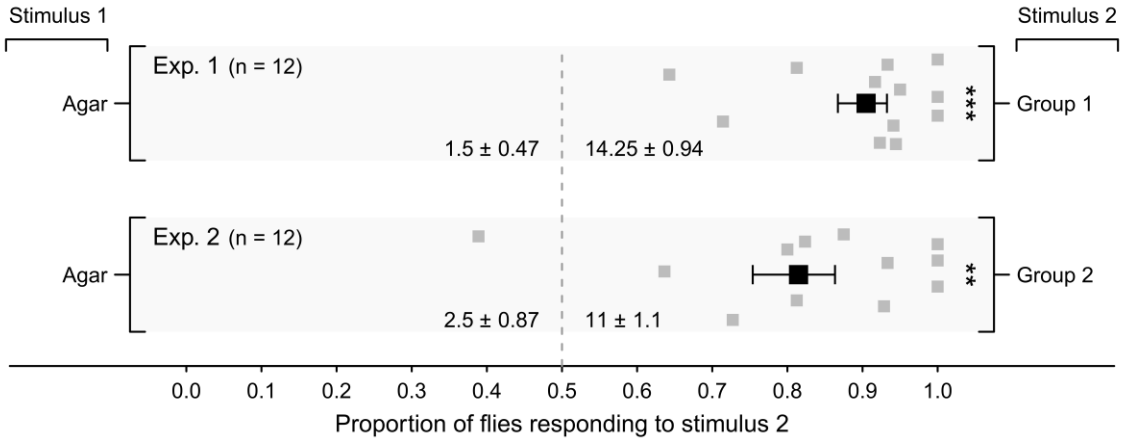

Figure 3.2 Mean (± SE) proportions of female stable flies captured in lateral chambers of still-air olfactometers (Figure 3.1A). Control chambers were baited with a plate of four (Exp. 1) or three (Exp. 2) sterile agar slices (Stimulus 1), whereas treatment chambers were baited with a plate of four (Exp. 1) or three (Exp. 2) agar slices, each slice growing (*i*) *Staphylococcus chromogenes*, *S. sciuri, S. simulans* or *S. succinus* (Stimulus 2; Group 1; Exp. 1), or (*ii*) *Corynebacterium stationis*, *Glutamicibacter protophormiae* or *Wautersiella* sp. (Stimulus 2; Group 2; Exp. 2). For each experimental replicate, 20 blood- and water-deprived female flies were released into the central chamber of the olfactometer and given 24 h to enter lateral chambers. Grey symbols show the proportion of flies captured in individual replicates in response to stimulus 2, whereas the black symbol shows the mean. Mean numbers of flies captured in response to test stimuli in experiments 1 and 2 are listed above the x-axis; **P < 0.01, ***P < 0.001, as determined by a likelihood ratio test.

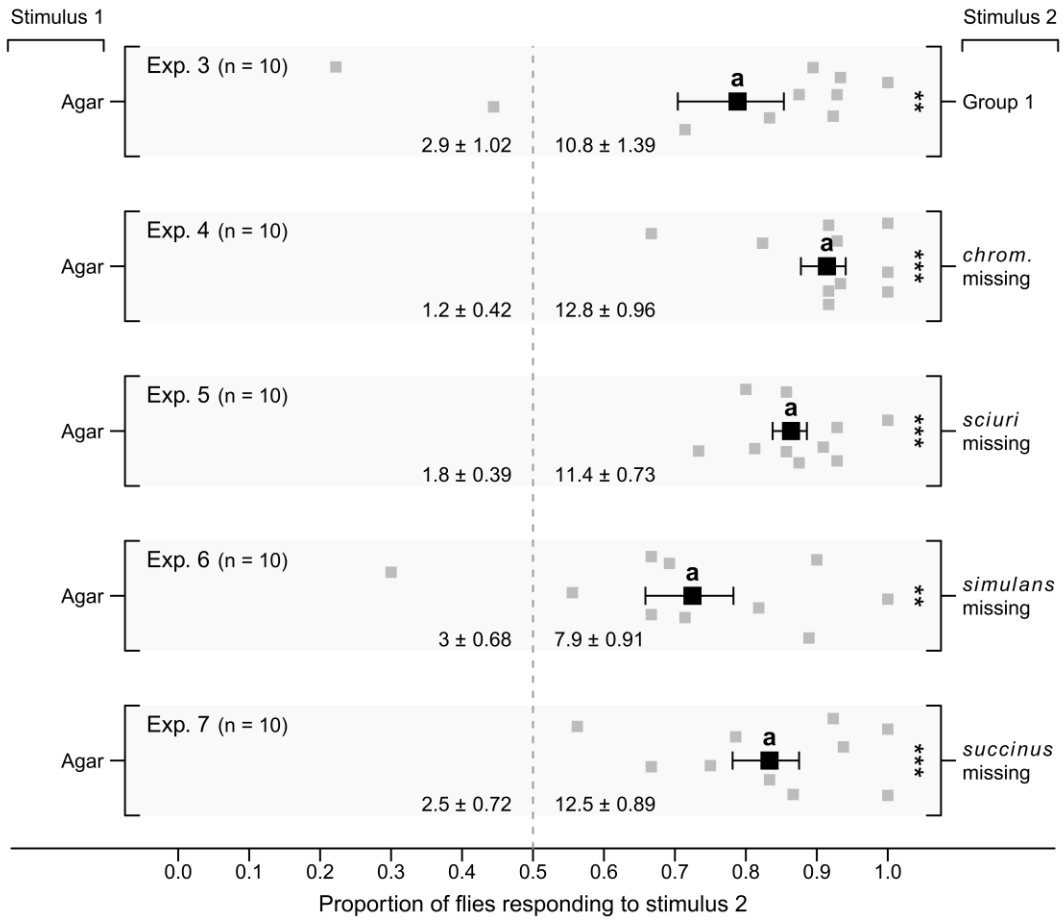

Figure 3.3 Mean (± SE) proportions of female stable flies captured in lateral chambers of still-air olfactometers (Figure 3.1A). Control chambers were fitted with a plate of four sterile agar slices (Stimulus 1, Exps. 3–7), whereas treatment chambers were baited with a plate of four agar slices, each slice growing (*i*) one of four *Staphylococcus* congeners (*S. chromogenes, S. sciuri, S. simulans,* or *S. succinus*) (Stimulus 2; Group 1; Exp. 3), or (*ii*) one of three *Staphylococcus* congeners, with one congener missing from Group 1 and one slice of sterile agar added (Stimulus 2; Exps. 4–7). For each experimental replicate, 20 blood- and water-deprived female flies were released into the central chamber of the olfactometer and given 24 h to enter lateral chambers. Grey symbols show the proportion of flies captured in stimulus-2 chambers in each replicate, whereas black symbols show the mean. Mean numbers of flies captured in response to test stimuli in each experiment are listed above the x-axis; *P < 0.05, **P < 0.01, ***P < 0.001, as determined by a likelihood ratio test; mean proportions in different experiments labelled with the same letter do not differ statistically, post-hoc Tukey tests, P > 0.05.

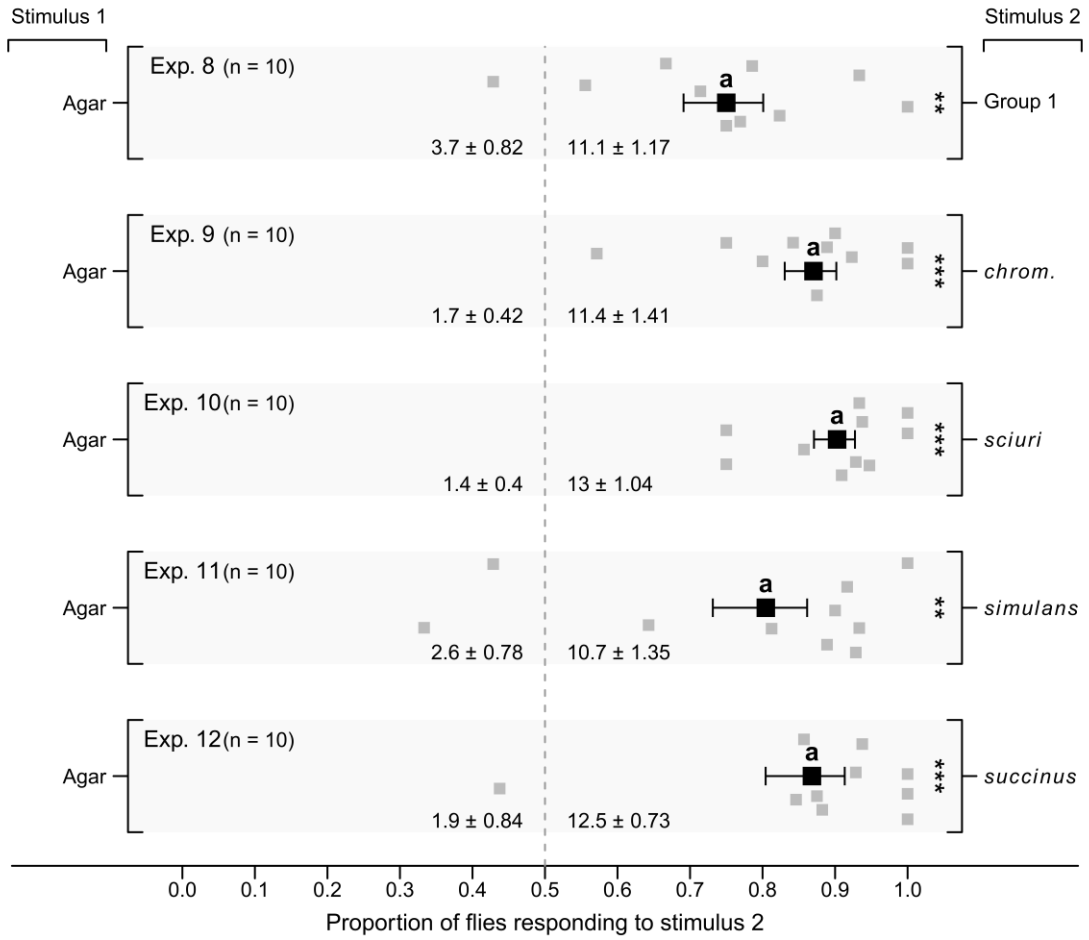

Figure 3.4 Mean (± SE) proportions of female stable flies captured in lateral chambers of still-air olfactometers (Figure 3.1A). Control chambers were fitted with a plate of four sterile agar slices (Stimulus 1, Exps. 8–12), whereas treatment chambers were baited with a plate of four agar slices, each slice growing (*i*) one of four *Staphylococcus* congeners (*S. chromogenes, S. sciuri, S. simulans,* or *S. succinus*) (Stimulus 2; Exp. 8), or (*ii*) one *Staphylococcus* congener, with three slices of sterile agar added (Stimulus 2; Exps. 9–12). For each experimental replicate, 20 blood- and water-deprived female flies were released into the central chamber of the olfactometer and given 24 h to enter lateral chambers. Grey symbols show the proportion of flies captured in stimulus-2 chambers in each replicate, whereas black symbols show the mean (± SE). Mean numbers of flies captured in response to test stimuli in each experiment are listed above the x-axis; **$P < 0.01$, ***$P < 0.001$, as determined by a likelihood ratio test; mean proportions in different experiments labelled with the same letter do not differ statistically, post-hoc Tukey tests, $P > 0.05$.

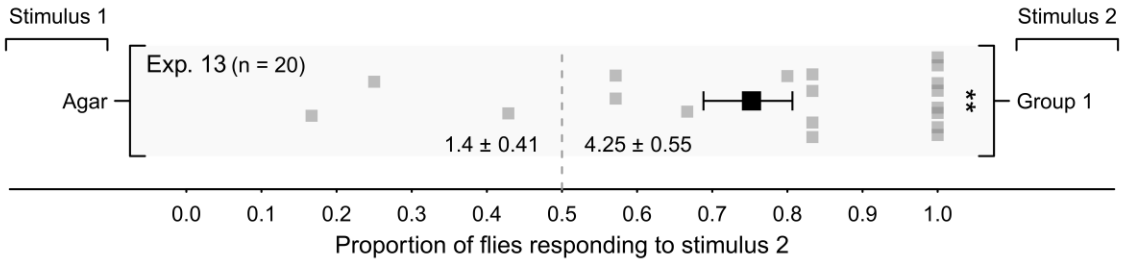

Figure 3.5 Mean (± SE) proportion of female stable flies captured on paired adhesive-coated cylindrical traps (Figure 3.1B) in a bioassay room. Control traps were fitted with a plate of four sterile agar slices (Stimulus 1), whereas treatment traps were baited with a plate of four agar slices, each slice growing separately one of four Staphylococcus congeners (*S. chromogenes*, *S. sciuri*, *S. simulans*, or *S. succinus*) (Group 1; Stimulus 2). For each experimental replicate, 20 blood- and water-deprived female flies were released into the room and given 24 h to respond. Grey symbols show the proportion of flies captured in individual replicates on stimulus-2 traps, whereas the black symbol shows the mean (± SE). Mean numbers of flies captured are listed above the x-axis; **P < 0.01, as determined by a likelihood ratio test.

Figure 3.6 Mean (± SE) proportion of female stable flies alighting on black barrels inside a greenhouse compartment (Figure 3.1C). Treatment barrels were baited with 3 agar plates growing *Staphylococcus sciuri* (Stimulus 2), whereas control barrels were fitted with 3 sterile agar plates (Stimulus 1). For each experimental replicate, 100 blood- and water-deprived female flies were released into the greenhouse compartment and given 10 min to respond. Grey symbols show the proportion of flies in each replicate alighting on stimulus-2 barrels, whereas the black symbol shows the mean (± SE). Mean numbers of alightings in response to test stimuli are listed above the x-axis; **$P < 0.01$, as determined by a likelihood ratio test.

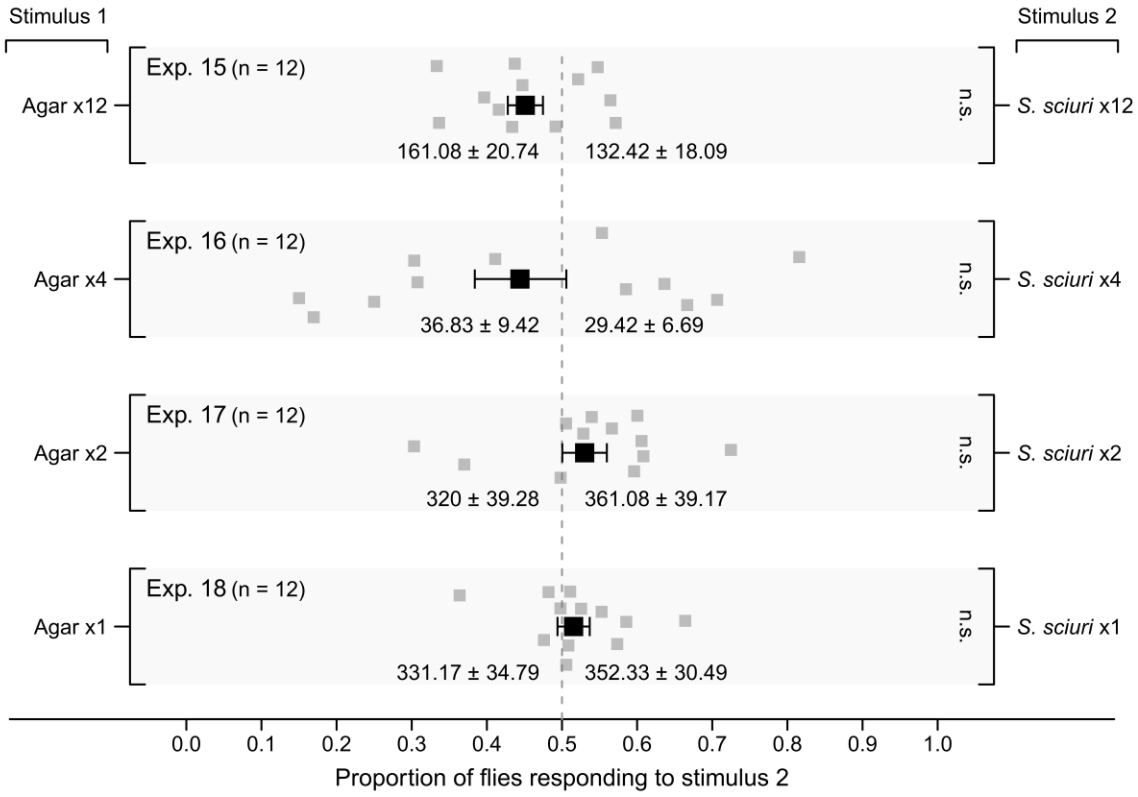

Figure 3.7 Mean (± SE) proportional alighting responses by wild stable flies on black barrels set up on a cattle farm (Figure 3.1D). Treatment barrels (Stimulus 2) were baited with 12 agar plates (Exp. 15), 4 plates (Exp. 16), 2 plates (Exp. 17) or 1 plate (Exp. 18) all inoculated with *Staphylococcus sciuri,* whereas control barrels (Stimulus 1) were fitted with corresponding numbers of sterile agar plates. For each experimental replicate, alighting responses by flies were video recorded for 5 min. Grey symbols show the proportion of alighting responses in each replicate and black symbols show the mean (± SE). Mean numbers of alightings in response to test stimuli are listed above the x-axis. There was no preference for Stimulus 2 in any experiment; n. s. = not significant.

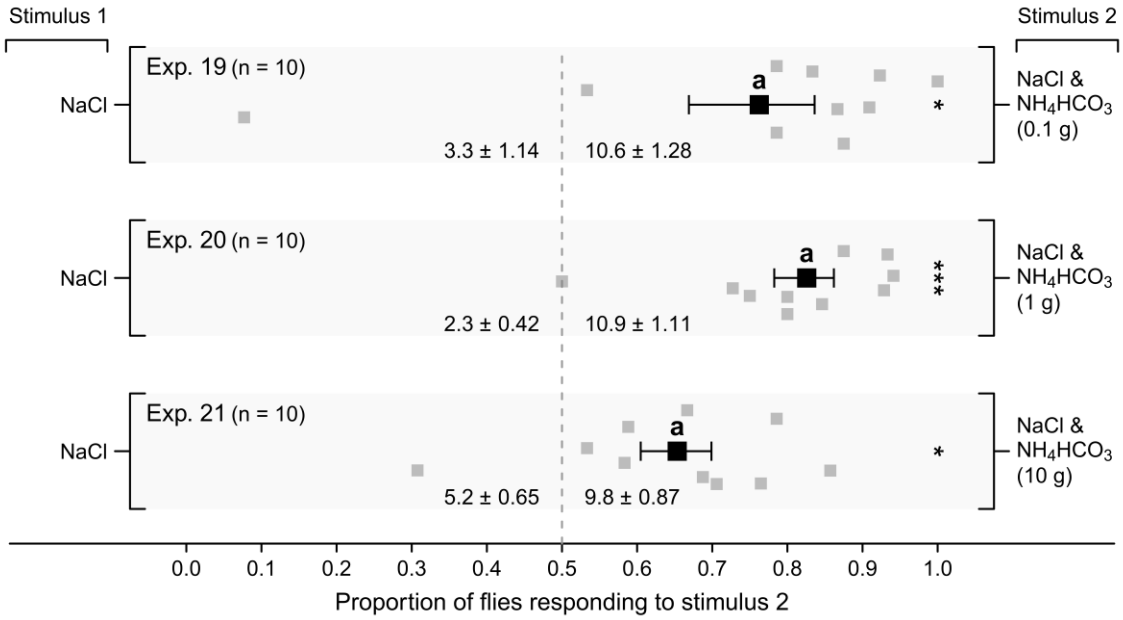

Figure 3.8 Mean (± SE) proportions of female stable flies captured in lateral chambers of still-air olfactometers (Figure 3.1E) baited with a sodium chloride solution (50 g NaCl in 50 mL water) (Stimulus 1; Exps. 19–21), or a sodium chloride solution also containing ammonium bicarbonate (NH_4HCO_3) at 0.1 g (Exp. 19, low dose), 1 g (Exp. 20, medium dose) or 10 g (Exp. 21, high dose), all emitting ammonia (NH_3). For each experimental replicate, 20 blood- and water-deprived female flies were released into the central chamber of the olfactometer and given 24 h to approach stimuli in lateral chambers. Grey symbols show the proportion of flies captured in individual replicates in response to stimulus 2, whereas the black symbols show the mean (± SE). Mean numbers of flies captured in response to test stimuli in each experiment are listed above the x-axis; *$P < 0.05$, ***$P < 0.001$, as determined by a likelihood ratio test. Mean proportions in different experiments labelled with the same letter do not differ statistically; post-hoc Tukey tests; $P > 0.05$.

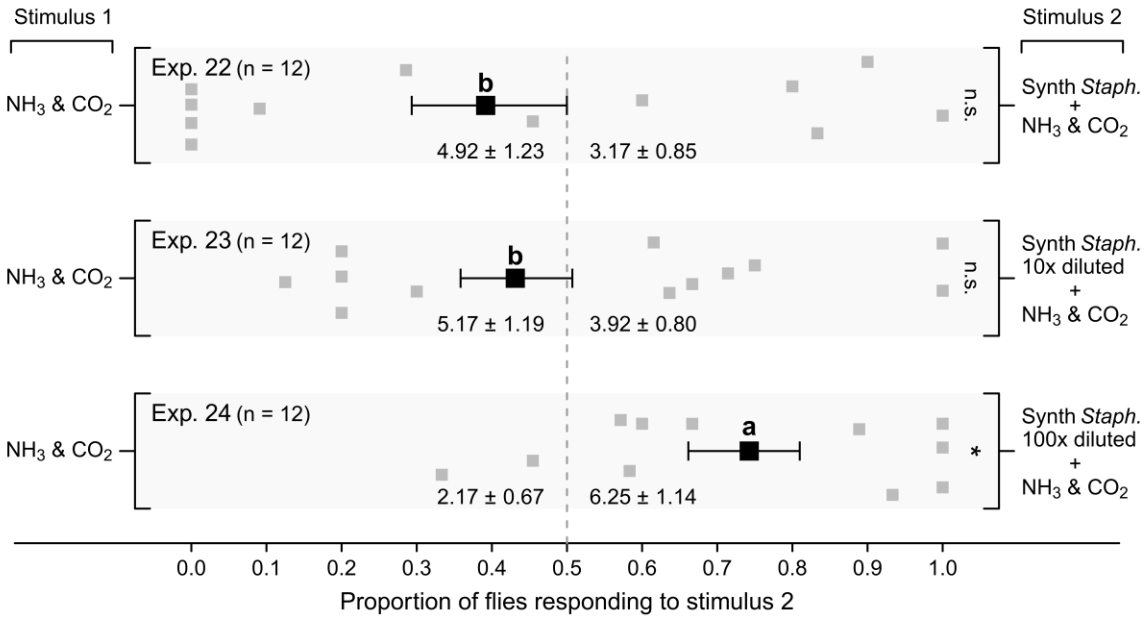

Figure 3.9 Mean (± SE) proportions of female stable flies captured in lateral chambers of still-air olfactometers (Figure 3.1E) baited with (*i*) the synthetic *Staphylococcus* blend ('Synth. *Staph*.') (Table 3.4) (Exp. 22), (*ii*) the blend 10× diluted ('Synth *Staph*. 10× diluted') (Exp. 23), or (*iii*) the blend 100× diluted ('Synth. *Staph*. 100× diluted') (Exp. 24). All three Stimulus 2 blends were formulated in mineral oil, whereas plain mineral oil served as the corresponding control stimulus. Present in both treatment and control chambers of all experiments was a sodium chloride (NaCl) and ammonium bicarbonate (NH_4HCO_3) solution (50 g NaCl and 0.1 g NH_4HCO_3 in 50 mL water) emitting ammonia (NH_3) and carbon dioxide (CO_2). For each experimental replicate, 20 blood- and water-deprived female flies were released into the central chamber of the olfactometer and given 24 h to approach stimuli in lateral chambers. Grey symbols show the proportion of flies captured in individual replicates in response to stimulus 2, whereas the black symbols show the mean (± SE). Mean numbers of flies captured in response to test stimuli in each experiment are listed above the x-axis; *$P < 0.05$, as determined by a likelihood ratio test. Mean proportions in different experiments labelled with the same letter do not differ statistically; post-hoc Tukey tests; $P > 0.05$.

Table 3.1 Summary of experiments (Exp.) and number of replicates (n) run, numbers of flies tested per replicate (flies/n), the bioassay scale (still-air 2-choice olfactometer, room, greenhouse, field; see Figure 3.1), and the stimuli tested.

Exp. # (n)	Flies/ n	Bioassay scale	Stimulus 1	Stimulus 2
Testing of select microbes for their attractiveness to flies				
1 (12)	20	Olfactometer[1]	Agar control	*Staphylococcus* congeners (Group 1)[5]
2 (12)	20	Olfactometer[1]	Agar control	Microbe heterogeners (Group 2)[6]
3 (10)	20	Olfactometer[1]	Agar control	Group 1
4 (10)	20	Olfactometer[1]	Agar control	Group 1 minus *S. chromogenes*
5 (10)	20	Olfactometer[1]	Agar control	Group 1 minus *S. scirui*
6 (10)	20	Olfactometer[1]	Agar control	Group 1 minus *S. simulans*
7 (10)	20	Olfactometer[1]	Agar control	Group 1 minus *S. succinus*
8 (10)	20	Olfactometer[1]	Agar control	Group 1
9 (10)	20	Olfactometer[1]	Agar control	*S. chromogenes*
10 (10)	20	Olfactometer[1]	Agar control	*S. sciuri*
11 (10)	20	Olfactometer[1]	Agar control	*S. simulans*
12 (10)	20	Olfactometer[1]	Agar control	*S. succinus*
13 (20)	20	Room[2]	Agar control	Group 1
14 (10)	100	Greenhouse[3,4]	Agar control (×3)	*S. sciuri* (×3)
15 (10)	N/A	Field[4]	Agar control (×12)	*S. sciuri* (×12)
16 (10)	N/A	Field[4]	Agar control (×4)	*S. sciuri* (×4)
17 (10)	N/A	Field[4]	Agar control (×2)	*S. sciuri* (×2)
18 (10)	N/A	Field[4]	Agar control (×1)	*S. sciuri* (×1)
Investigation of mechanisms underlying attraction of flies to Staphylococcus spp.				
19 (20)	10	Olfactometer	NaCl solution[7]	NaCl solution[7] & 0.1 g NH_4HCO_3
20 (20)	10	Olfactometer	NaCl solution[7]	NaCl solution[7] & 1 g NH_4HCO_3
21 (20)	10	Olfactometer	NaCl solution[7]	NaCl solution[7] & 10 g NH_4HCO_3
22 (12)	20	Olfactometer	NaCl & NH_4HCO_3 solution[8] & mineral oil	NaCl & NH_4HCO_3 solution[8] & synthetic *Staphylococcus* blend[9]
23 (12)	20	Olfactometer	NaCl & NH_4HCO_3 solution[8] & mineral oil	NaCl & NH_4HCO_3 solution[8] & synthetic *Staphylococcus* blend[9] (10× dilution)
24 (12)	20	Olfactometer	NaCl & NH_4HCO_3 solution[8] & mineral oil	NaCl & NH_4HCO_3 solution[8] & synthetic *Staphylococcus* blend[9] (100× dilution)
25 (10)	20	Room	NaCl & NH_4HCO_3 solution[8] & mineral oil	NaCl & NH_4HCO_3 solution[8] & synthetic *S. sciuri* blend[9]

[1]olfactometer dimension: 46 × 21.5 × 15.5 cm (Figure 3.1A); [2]room dimension: 225 × 230 × 230 cm; [3]greenhouse dimension: 600 × 600 × 360 cm (Figure 3.1C); [4]test stimuli were presented with paired black barrels as surrogate host objects; [5]Group 1: *S. sciuri, S. succinus, S. simulans, S. chromogenes;* [6]Group 2: *Glutamicibacter protophormiae, Corynebacterium stationis, Wautersiella* sp.; [7]2.5 mL of NaCl solution (50 g NaCl dissolved in 50 mL water); [8]2.5 mL of solution prepared by dissolving 50 g NaCl and 0.1 g NH_4HCO_3 in 50 mL water; [9]Table 3.4

Table 3.2 List of microbes isolated from the skin of a live calf and/or the hide of a recently slaughtered adult cow and identified to the genus and/or species level.

Species	**Species**
Acinetobacter baumanni	*Klebsiella aerogenes*
Acinetobacter gerneri	*Klebsiella pneumonia*
Acinetobacter johnsonii	*Kurthia gibsonii*
Acinetobacter proteolyticus	*Kurthia populi*
Acinetobacter sp.	*Lampropedia aestuarii*
Acinetobacter variabilis	*Pantoea agglomerans*
Alcaligenes faecalis	*Proteus mirabilis*
Bacillus pumilis	*Pseudochrobactrum asaccharolyticum*
Bacillus subtilis	*Pseudomonas aeruginosa*
Burkholderia multivorans	*Serratia marcescens*
Candida catenulate	*Staphylococcus chromogenes*
Citrobacter koseri	*Staphylococcus sciuri*
Corynebacterium glutamicum	*Staphylococcus simulans*
Corynebacterium stationis	*Staphylococcus succinus*
Enterobacter cloacae	*Stenotrophomonas maltophilia*
Enterobacter hormaechei	*Stenotrophomonas pavanii*
Enterobacter kobei	*Stenotrophomonas* sp.
Escherichia coli	*Wautersiella* sp.
Glutamicibacter protophormiae	*Wickerhamomyces anomalus*

Table 3.3 List of volatiles identified, and ppm ammonia measured, in the headspace of four *Staphylococcus* microbes attractive to stable flies.

Compounds (% purity) & ammonia	*S. chromogenes*	*S. sciuri*	*S. simulans*	*S. succinus*
3-methyl-1-butenol (97)[1]		✓	✓	
isoamyl alcohol (95)[2]	✓	✓	✓	✓
3-methyl-butanoic acid (99)[1]		✓	✓	✓
2-methyl-butanoic acid (98)[1]		✓	✓	✓
isoamyl acetate (95)[3]	✓	✓	✓	✓
2,5-dimethyl pyrazine (98)[1]	✓	✓	✓	✓
3-methylbutyl-2-methylpropionate (99)[4]	✓			✓
trimethyl pyrazine (99)[1]	✓	✓	✓	✓
2-isopropyl-5-methyl-pyrazine (95)[5]	✓	✓	✓	✓
3-methylbutyl-2-methylbutyrate (95)[6]				✓
3-methylbutyl-3-methylbutyrate (95)[7]	✓			✓
2-ethyl-3,5(6)-dimethylpyrazine (99)[8, 9]	✓	✓	✓	✓
2-phenylethyl acohol (99)[10]	✓	✓	✓	✓
benzene acetonitrile (98)[1]	✓			✓
2-phenylethyl-iso-butyrate (95)[11]	✓			✓
ammonia (NH_3)	8.0 ± 1.6 ppm	14.3 ± 2.6 ppm	13.0 ± 0.0 ppm	9.3 ± 0.9 ppm

[1]Sigma-Aldrich; [2]Fischer; [3]acetylated from the alcohol using acetic anhydride; [4]esterified (Nieses et al. 1978) from isoamyl alcohol[1] and isobutyric acid[1]; [5]synthesized as previously described (Masuda et al. 1981; Mihara & Masuda 1990); [6]esterified (Nieses et al. 1978) from isoamyl alcohol[1] and 2-methyl butyric acid[1]; [7]esterified (Nieses et al. 1978) from isoamyl alcohol[1] and 3-methyl butyric acid[1]; [8]Acros; [9]composed of 50% 2-ethyl-3,5-dimethylpyrazine and 50% 2-ethyl-3,6-dimethylpyrazine; [10]Fluka; [11]esterified (Nieses et al. 1978) from phenylethyl alcohol[1] and isobutyric[1]

Table 3.4 Preparation of synthetic blends of odorants (i) shared between the four Staphylococcus species (Synthetic *Staph*.) or (ii) present in the headspace of *S. sciuri* (Synthetic *S. sciuri*). Three 1-dram vials ([1,2,3]) were used to release groups of chemicals. The proportion of each chemical in each group is presented.

		Synthetic *Staph* **(Exp. 22)**	**Synthetic *S. sciuri*** **(Exp. 25)**
Compound #	**Compound name**	**% in mix**	**% in mix**
1[1]	3-methylbutenol	3.7	3.15
2[1]	isoamyl alcohol	59.3	94.5
3[1]	3-methyl butanoic acid	18.5	1.57
4[1]	2-methyl butanoic acid	18.5	0.787
5[2]	isoamyl acetate	0.34	0.88
6[2]	2,5-dimethylpyrazine	47.8	88.5
7[2]	trimethyl pyrazine	17.1	4.4
8[2]	3-methylbutyl-2-methyl propionate	0	0.88
9[2]	2-isopropyl-5-methyl pyrazine	27.3	0.88
10[2]	2-ethyl-3,5(6)-dimethyl pyrazine[4]	6.8	0.88
11[2]	3-methylbutyl-2-methyl butyrate	0	1.76
12[2]	3-methylbutyl-3-methyl butyrate	0	0.88
13[2]	2-phenylethyl-iso-butyrate	0	0.88
14[2]	benzene acetonitrile	0.68	0
15[3]	2-phenylethyl alcohol	100	100

[1]5 µL of a mix of compounds 1–4 in their listed proportions was added to 1 mL of mineral oil, of which 50 µL were then added to an uncapped 1-dram glass vial for use in a single bioassay replicate.
[2]a mix consisting of 2.5 µL (Exp. 22) or 5 µL (Exp. 25) of compounds 5–14 in their listed proportions was added to 1 mL of mineral oil, of which 50 µL were then added to an uncapped 1-dram glass vial for use in a single bioassay replicate.
[3]50 µL of compound 15 was pipetted into a 1-dram glass vial, the lid of which was opened approximately 34°.
[4]Composed of 50% 2-ethyl-3,5-dimethylpyrazine and 50% 2-ethyl-3,6-dimethylpyrazine

Chapter 4: Blood-feeding stable flies, *Stomoxys calcitrans*, are attracted to, and transmit *Staphylococcus aureus*, a causal agent of bovine mastitis – a laboratory pilot study

A near identical version of this chapter has been submitted to *Journal of Medical Entomology* with the following authors: Saif Nayani, Sanam Meraj, Asim Renyard & Gerhard Gries. Manuscript ID: JME-2023-0401

Abstract

Stable flies, *Stomoxys calcitrans* (Diptera: Muscidae), are blood-feeding ectoparasites of cows and thus potential vectors of the skin-dwelling bacterium *Staphylococcus aureus*, a causal agent of bovine mastitis which inflicts udder inflammation in cows. Our objectives were to determine whether stable flies (1) are attracted to disease-causing strains of *S. aureus*, and (2) transmit *S. aureus* from infected blood to sterile blood. In 3-chamber olfactometers, five of eight *S. aureus* strains grown on agar and tested versus sterile agar attracted female stable flies. When flies ingested droplets of blood inoculated with *S. aureus* at doses of 0 (control), 10^5 (low), 10^7 (medium) and $>10^9$ (high) colony forming units per milliliter and subsequently ingested sterile blood, they transmitted *S. aureus* to the sterile blood. The dose of *S. aureus* in blood droplets fed upon by flies during their first feeding bout dose-dependently affected the amount of bacteria that flies transmitted to sterile blood during their second feeding bout, but the time elapsed between feeding bouts (0 h, 1 h, 8 h and 24 h) had no effect on the amount of microbes transmitted to sterile blood. Our data infer the existence of a positive feedback loop. First, stable flies carrying *S. aureus* and feeding on cows transmit *S. aureus*, thereby causing mastitis. As *S. aureus* bacteria of afflicted cows proliferate, they attract even more flies which, in turn, worsen the infection. This type of feedback loop underscores the need for effective stable fly control tactics that curtail the incidence of bovine mastitis in cows.

Keywords: stable flies, cows*, Staphylococcus aureus,* attraction, bacterial transmission, host-foraging, bovine mastitis

4.1) Introduction

Hematophagous insects transmit a plethora of disease-causing pathogens to their vertebrate hosts. For examples, fleas (Siphonaptera) transmit the bacterium *Yersinia pestis* which causes plague (Wimsatt and Biggins 2009); kissing bugs (Hemiptera: Reduviidae), particularly *Triatoma infestans*, *Rhodnius prolixus*, and *Panstrongylus megistus*, transmit *Trypanosoma cruzi* which causes Chagas disease (Steverding 2014); blackflies (Diptera: Simuliidae) transmit the parasitic nematode *Onchocerca volvulus* which causes river blindness (Onchocerciasis) (Hougard et al. 1997); and tsetse (Diptera: Glossinidae) transmit *Trypanosoma brucei* which causes sleeping sickness (Malvy and Chappuis 2011). Mosquitoes (Diptera: Culicidae) alone transmit many pathogens to their vertebrate hosts that cause deadly and debilitating diseases. For examples, *Aedes* spp. Mosquitoes transmit viruses which cause Chikungunya (Lounibos and Kramer 2016), Dengue (Weetman et al. 2018), Rift Valley fever (*Phlebovirus*) (Kwasnik et al. 2021), yellow fever (Barrett and Higgs 2007), and Zika (Rabaan et al. 2017). Similarly, *Culex* spp. mosquitoes transmit the Rift Valley Fever virus (Kwasnik et al. 2021) and the West Nile Fever virus (Hayes 2001) as well as the parasitic nematode *Wuchereria bancrofti* which causes lymphatic filariasis (Manguin et al. 2010). Finally, *Anopheles* spp. Mosquitoes transmit both *W. bancrofti,* and the *Plasmodium* that causes Malaria (Manguin et al. 2010).

Stable flies, *Stomoxys calcitrans* (Diptera: Muscidae), are ectoparasites of livestock, particularly cattle. Their blood-feeding activity diminishes the feed efficiency of cattle, slows their weight gain, lowers milk production, and ultimately reduces revenues for life stock industries (Bruce and Decker 1958; Campbell et al. 1977; Campbell et al. 2001; Taylor et al. 2012). Previous studies have investigated whether stable flies mechanically transmit pathogens which cause polio (Anderson and Frost 1912, 1913; Rosenau and Brues 1912; Sawyer and Herms 1913), paratyphoid (Birk 1932), Leishmaniasis (Berberian 1938; Lainson and Southgate 1965), Yellow Fever (Hoskins 1934), Tularemia (Olsufiev 1940), African Swine Fever (Mellor et al. 1987), and even Plague and Plague-like diseases (Wayson 1914) but results were often inconclusive. More recent data on pathogen transmission by stable flies are more conclusive but conflicting reports still exist. When stable flies had ingested blood inoculated with *Enterobacter sakazakii*

bacteria, they transmitted them to sources of sterile blood and honey-water for at least 20 days (Mramba et al. 2007). Stable flies that had fed on a hamster infected with the Rift Valley Fever virus at a biologically realistic viral load of $10^{9.7}$ plaque-forming units (PFUs) per mL, transmitted the virus to healthy hamsters in 57% of subsequent feeding bouts (Turell et al. 2010). Stable flies that sequentially fed on blood infected with West Nile Virus (WNV), and then on sterile blood, transmitted WNV and WNV-RNA in 6% and 26.5%, respectively, of all feeding trials (Doyle et al. 2011). When stable flies first fed on blood infected with the arterivirus that causes porcine reproductive and respiratory syndrome in pigs, and then fed on healthy pigs, they failed to transfer the arterivirus (Rochon et al. 2011). Moreover, stable flies were found to not carry the spiral-shaped bacterium *Treponema phagedenis* (Thibodeaux et al. 2021) which is deemed a causal agent of bovine digital dermatitis. Conversely, 11.3% of wild stable flies carried the gram-negative bacterium *Anaplasma marginale*, the causative agent of bovine anaplasmosis (Araujo et al. 2021). Outbreaks of viral lumpy skin disease in bovines were correlated with high abundance of stable flies, implying a vectorial function of flies (Kahana-Sutin et al. 2017). Finally, modelling transmission of the African Swine Fever virus on a pig farm revealed that increasing stable fly loads (from 5–10 to 50–100 flies per pig) increased viral transmission from 10–18% to 48–64% (Vergne et al. 2021).

Bovine mastitis (henceforth 'mastitis') is a painful inflammation of the udder typically caused by bacterial infections. Depending upon the class of infection (clinical, sub-clinical or chronic), symptoms range from swollen udders, fevers, watery or clotted milk, to death (Cheng and Han 2020 & references therein). Regardless of the infection class, infected cows commonly produce less milk, resulting in lost revenue of $177 USD per cow per year in Northwestern Europe and Canada (Hogeveen et al. 2019). In Ethiopia, revenue losses amounted to $29 per cow per year but the impact was far higher when adjusted for relative incomes between regions (Hogeveen et al. 2019). Approximately 58%, 26% and 17% of these losses were attributed to lower milk production, culling of affected cows, and veterinary costs, respectively (Hogeveen et al. 2019). In the US, mastitis-caused revenue losses were estimated to be $72 per cow per year which – when multiplied by the 8.7 million cows in the US – add up to $629 million (Hogeveen et al. 2019).

Infection with *Staphylococcus aureus* is a cause of mastitis (Zhao and Lacasse 2008). Besides the 'typical' mastitis symptoms (see above), infection of mammary tissues with specifically *S. aureus* also causes necrosis of milk-producing cells and their replacement with non-secretory cells (Zhao and Lacasse 2008), ultimately lowering milk production. As mastitis is caused by bacterial infection (Zhao and Lacasse 2008 and references therein), the mode of bacteria transmission ought to be investigated. In DNA 'fingerprint analyses' for the presence of *S. aureus* bacteria, they were confirmed in samples from ectoparasitic horn flies, *Haematobia irritans* (Diptera: Muscidae), heifer mammary secretions, and heifer streak canals (where milk passes through the teat) (Gillespie et al. 1999). Noteworthy, the same two bacterial strains isolated from flies were found in all but three heifer samples, suggesting that horn flies transmit *S. aureus* to cows. Furthermore, when the teats of healthy cows were exposed to horn flies carrying *S. aureus*, intra-mammary infections occurred in three out of four trials (Owens et al. 1998). Lastly, scabs of heifers naturally infected with *S. aureus* contained high concentrations of these bacteria (Owens et al. 1998). Altogether, these results provide incentive to explore whether not only horn flies, but also stable flies, transmit mastitis-causing *S. aureus* to their vertebrate blood hosts.

Drawing on findings that stable flies are attracted to *Staphylococcus* bacteria (Nayani et al. 2023a), our objectives here were to determine whether stable flies (1) are attracted to disease-causing strains of *S. aureus,* and (2) transmit *S. aureus* from infected blood to sterile blood. If so demonstrated, there would be emerging evidence for a positive feedback loop in which *S. aureus*-infected cows strongly attract stable flies which, in turn, then increases the cows' infection load and thus their level of attractiveness to foraging stable flies.

4.2) Materials and Methods

4.2.1) Rearing of experimental flies

Flies were reared as described (Nayani et al. 2023a,b). Briefly, flies were kept in mesh cages (45 × 45 × 45 cm) in a hyperbolic growth chamber (BioChambers Inc., Winnipeg, MB, CA) on the Burnaby campus of Simon Fraser University. Twice per day, flies were fed with citrated bovine blood, and three times per week, they were provided with a wet

black cloth as an oviposition site. Eggs were then transferred to a larval rearing medium (Friesen et al. 2018, Nayani et al. 2023a,b). Flies were separated by sex (Nayani et al. 2023a,b), and only 7- to 11-day-old female flies, which responded better than male flies in similar studies (Nayani et al. 2023a,b), were used in experiments.

4.2.2) Acquisition and maintenance of *Staphylococcus aureus*

Eight strains of *S. aureus* (S313, S323, S340, S369, S383, W512, W517, W533) had been isolated from cows with bovine mastitis (Anderson and Lyman 2006; Anderson et al. 2012) and were provided by the Anderson-laboratory at the University of North Carolina. Stock plates of these strains were maintained on Mueller Hinton Agar at 4 °C.

4.2.3) O1: Determine whether stable flies are attracted to disease-causing strains of *S. aureus*

To determine whether stable flies are attracted to strains of *S. aureus,* bioassays were run in 3-chamber olfactometers (Figure 4.1), adopting the protocol previously detailed (Nayani et al. 2023a). To initiate experimental replicates, treatment and control stimuli were placed in the lateral chambers of olfactometers. In each of nine parallel experiments (n = 10 each), treatment and control stimuli were presented in sterile Petri dishes. The treatment stimulus consisted of three quarter-slices of sterile agar and one quarter-slice of agar inoculated, and grown overnight, with a single strain of *S. aureus* [S313 (Exp. 1), S323 (Exp. 2), S340 (Exp. 3), S369 (Exp. 4), S383 (Exp. 5), W512 (Exp. 6), W517 (Exp. 7), W533 (Exp. 8)], or with *Staphylococcus sciuri* (Exp. 9; positive control) which is proven attractive to stable flies (Nayani et al. 2023a). Corresponding control stimuli in each experiment consisted of four sterile quarter-slices of agar. Bioassays were terminated after 24 h by placing olfactometers in a freezer (-15 ℃), and then counting cold-euthanized flies in each lateral chamber. Flies remaining in the central chamber were deemed non-responders.

4.2.4) O2: Determine whether stable flies transmit *S. aureus* from infected blood to sterile blood

This experiment was designed to determine whether stable flies that sequentially feed on blood infected with *S. aureus,* and then on sterile blood, transmit *S. aureus* to the sterile blood. Sterile blood (Hemostat Laboratories, Dixon, CA, USA via Cedar Lane, Burlington, NC, USA) was infected with *S. aureus* to reach concentrations of colony-forming units per microlitre (CFU/mL) of 10^5 (low), 10^7 (medium) and $>10^9$ (high). To this end, a liquid culture of *S. aureus* was grown overnight, and then diluted to a stock culture with a dose of 10^8 CFU/mL. Bacterial concentrations were determined by optical density ($OD_{600\ nm}$) readings with a spectrophotometer (Molecular Devices, SJ, CA, USA). OD values were then converted to bacterial concentrations, and once the concentration of the stock solution was determined, it was diluted to 10^8 CFUs/mL. One part of this stock culture was combined with (*i*) nine parts of sterile blood to form the medium-dose blood sample (10^7 CFU/mL), or with (*ii*) 999 parts of sterile blood to form the low-dose blood sample (10^5 CFU/mL). The high-dose blood sample ($>10^9$ CFU/mL) was prepared by scraping a large section of *S. aureus* from a stock plate to a sterile blood sample. Finally, a sterile control blood sample (0 CFU/mL) was used to determine whether laboratory flies carry *S. aureus* and are capable of transmitting it to sterile blood.

To initiate a feeding trial, each of three 24-h blood- and water-deprived flies was gently pushed, head-first, into a 200-µL pipette-tip (Corning, Corning, NY, USA) adapted as 'fly restraint' until her head (but no legs) protruded from the cut tip of the restraint (Figure 4.2a). Three fly restraints, each housing one fly, were hot-glued 3.5 cm apart on a popsicle stick (Crafts via Dollarama, Montreal, QC, CA) (Figure 4.2b). After three 20-µL droplets of blood were pipetted onto the lid of a sterile petri dish (Figure 4.2c), the three fly restraints were lowered such that the flies could feed on the blood droplets (Figure 4.2d). After 20 s of feeding, the restrains were lifted for 10 s to gauge the amount of blood remaining. This procedure was run 6× for a total of 3 min per feeding trial. A set of three flies was used for each dose of *S. aureus* bacteria (zero/control, low, medium, high) in the blood. After the flies' first feeding bout, they were removed from fly restraints and placed in mesh-covered jars (h = 9 cm; d = 6 cm) until their second feeding bout exclusively on sterile blood, starting 0 h, 1 h, 8 h, and 24 h after their first

feeding bout. In total, 16 experiments were run across the different bacterial doses (zero/control, low, medium, high) and time points (0 h, 1 h, 8 h, 24 h).

Upon completion of each second feeding bout, 15 µL of the remaining fed-upon blood of each of the three droplets per trial was pipetted onto, and spread over, the surface of a plate of Mannitol Salt Agar (MSA), a selective-differential growth medium for *S. aureus* (Chapman 1945). The presence of bacteria turns the agar from red to yellow, thus allowing for (tentative) identification of *S. aureus* in samples. The plates were left overnight in a 37 °C incubator and checked for bacterial growth the following day. If growth was detected, colonies were counted. To ensure that they were *S. aureus* colonies, one random colony from each sample with bacterial growth was selected to be regrown overnight at 37 °C on Mueller Hinton Agar. The most isolated single colony was then indicated on the bottom of the plate (Long et al. 2023) which was sent to Genewiz (Seattle, WA, USA) for 16SrRNA gene sequence amplification through polymerase chain reaction. FASTA sequences provided by Genewiz were compared with those in the National Center for Biotechnology Information's National Library of Medicine's standard sequence database, using the National Library of Medicine's Basic Local Alignment Search Tool to verify that sent samples were indeed *S. aureus*. A sequence was considered a match, if there was at least 95% coverage and 99% identity between the sequenced isolate and a known sequence (Nayani et al. 2023a; Long et al. 2023).

4.2.5) Statistical analyses

Data of bioassay experiments that tested attraction of stable flies to strains of *S. aureus* were analyzed with binomial generalized linear models (GLMs), using quasibinomial errors to account for overdispersion (RStudio v4.1.1) (Nayani et al. 2023b; Crawley 2007; RStudio Team 2023). These analyses compared an intercept-only model to a null model with a likelihood ratio test to determine whether the proportions of flies responding to treatment stimuli differed from a hypothetical 0.5 proportion. To test for differences in proportions among experiments 1–9 which shared a common control stimulus, similar generalized linear models with data from multiple experiments were created. Models with an individual intercept for each experiment were compared to a

model with a single intercept, again with a likelihood ratio test (Hothorn et al. 2008; Nayani et al. 2023c).

Data of *S. aureus* transmission experiments were analyzed using a GLM, using a negative binomial distribution. As there was no bacterial transmission when flies fed on sterile blood samples during their first and second feeding bout, we omitted these '0-data' from our model to improve model fit. Initially, we fit numbers of colonies as the response variable, and used bacterial dose (zero/control, low, medium, high), time elapsed between first and second feeding bout (0 h, 1 h, 8 h, 24 h), and their interaction as predictor variables. Inspection of this model, compared to a model containing treatment only as a predictor, indicated that bacterial dose alone offered better explanatory power based on model AIC. Consequently, we proceeded using a model using bacterial dose only as a predictor. We evaluated the significance of bacterial dose using a likelihood ratio test and conducted Tukey adjusted pairwise comparisons between bacterial doses. A p-value of < 0.05 was considered significant in all experiments (Nayani et al. 2023c).

4.3) Results

4.3.1) O1: Determine whether stable flies orient toward disease-causing strains of *S. aureus*

Of the *S. aureus* strains tested, five attracted significantly more flies than sterile agar controls (Figure 4.3), including S313 (Exp. 1; $F = 11.34$, $p < 0.05$), S323 (Exp. 2; $F = 627.67$, $p < 0.05$), S340 (Exp. 3; $F = 5.29$, $p < 0.05$), S383 (Exp. 5; $F = 21.73$, $p < 0.05$), and W517 (Exp. 7; $F = 7.68$, $p < 0.05$), as did *S. sciuri* (Exp. 9; $F = 8.92$, $p < 0.05$) which was tested as a positive control. Conversely, three strains were not more attractive than sterile agar controls (Figure 4.3), including S369 (Exp. 4; $F = 3.13$, $p > 0.05$), W512 (Exp. 6; $F = 0.21$, $p > 0.05$), and W533 (Exp. 8; $F = 0.46$, $p > 0.05$). There were no significant differences between the proportions of flies attracted to bacterial strains across experiments ($F = 1.32$, $df = 8$, $p > 0.05$; Figure 4.3).

4.3.2) O2: Determine whether stable flies transmit *S. aureus* from infected to sterile blood

When flies fed on sterile blood in both their first and second feeding bout, no bacterial transmission occurred. Conversely, bacterial transmission did occur for all bacterial doses tested (number of colonies formed on MSA after second feeding bout; back-transformed estimated marginal means ± SE: low dose: 1.33 ± 0.41; medium dose: 21.83 ± 8.51; high dose: 38.25 ± 10.36; Figure 4.4). The dose of *S. aureus* in blood samples (zero/control, low, medium, high) that flies ingested during their first feeding bout significantly affected the number of bacterial colony-forming units (CFUs) in sterile blood after being fed on by flies during their second feeding bout ($\chi^2 = 18.06$, df = 2, $p < 0.05$), indicating that the amount of bacterial transmission by flies was dependent upon the bacterial dose they had previously ingested. Ingestion of a medium or high bacterial dose by flies resulted in significantly more CFUs in fed-on sterile blood than ingestion of a low bacterial dose (high *vs* low dose: $z = 8.22$, $p < 0.05$; medium *vs* low dose: $z = -5.64$, $p < 0.05$). The numbers of CFUs appearing in sterile blood after being fed on by flies that had ingested a medium or a high bacterial dose did not differ (medium *vs* high dose: $z = 1.18$, $p > 0.05$).

4.4) Discussion

Our data show that stable flies are attracted to disease-causing strains of *S. aureus,* and that they transmit *S. aureus* from infected blood to sterile blood.

The potential role of stable flies as vectors of disease-causing pathogens has been investigated in diverse studies (Mramba et al. 2007; Turell et al. 2010; Doyle et al. 2011; Rochon et al. 2011; Kahana-Sutin et al. 2017; Vergne et al., 2021; Araujo et al. 2021; Thibodeaux et al. 2021) but the ability of stable flies to transmit *S. aureus* as a causal agent of bovine mastitis has hardly been investigated. As a non-motile pathogen, *S. aureus* requires transmission to new hosts through various means such as physical contact with contaminated sources or insect vectors including stable flies or horn flies (Chirico et al. 1997). As reliance on chance encounters with potential insect vectors could lower the fitness of *S. aureus*, we predicted that *S. aureus* attracts stable flies, resulting in transportation to new hosts. This prediction was supported by previous findings that *S. aureus* congeners – *S. chromogenes, S. sciuri, S. simulans* and *S. succinus* – in the bovine

skin microbiome have already been shown to attract stable flies (Nayani et al. 2023a). Building on these findings and adopting the same experimental protocol, we tested attraction of stable flies to eight strains of *S. aureus* previously isolated from diseased cows (Anderson and Lyman 2006; Anderson et al. 2012), using proven-attractive *S. sciuri* (Nayani et al. 2023a) as a positive reference. That five out of these eight *S. aureus* strains indeed attracted stable flies suggests that *S. aureus* manipulates the behaviour of flies to achieve transportation between hosts. As CO_2 and odorant emissions from deer-associated microbes attract Western black-legged ticks, *Ixodes pacificus* (Ixodida: Ixodidae) (Long et al. 2023), we predict that the mechanisms underlying stable fly attraction to *S. aureus* are also microbe-produced semiochemical gases and odorants.

Ethical and animal welfare concerns prohibited '*in vivo'* experiments to test for transmission of *S. aureus* from mastitis-afflicted cows to healthy cows. Instead, we opted for a proof-of-concept experiment to demonstrate fly-mediated transmission of *S. aureus* from infected bovine blood to sterile bovine blood. To this end, we allowed flies at first to ingest droplets of blood inoculated with *S. aureus* at doses of 0 (control), 10^5 (low), 10^7 (medium) and $>10^9$ (high) CFUs/mL, and then to ingest sterile blood. Whenever flies had ingested blood infected with *S. aureus* irrespective of dose, they transmitted *S. aureus* to sterile blood. However, the dose of *S. aureus* in blood droplets (zero/control, low, medium, high) fed upon by flies during their first feeding bout dose-dependently affected the amount of microbes they transmitted to sterile blood during their second feeding bout. These results are important in that – generally – infections of new hosts and disease manifestations are contingent upon the concentration of the microbial inoculum that is transmitted (Rello et al. 2009). It follows that flies feeding on cows heavily afflicted with mastitis and carrying a large load of *S. aureus* are likely to transmit *S. aureus* to healthy cows. This inference is supported by a study showing that stable flies that had ingested blood infected with 10^7 plaque-forming units per milliliter (PFUs/mL) of the West Nile Virus (WNV) transmitted WNV RNA in 26.5% of all trials (Doyle et al. 2011). Similarly, flies that had fed on hamsters infected with the Rift Valley Fever virus at $>10^9$ PFUs/mL transmitted the virus to healthy hamsters in >50% of all trials (Turell et al. 2010). Interestingly, the time elapsed between the first and second feeding bout (0 h, 1 h, 8 h and 24 h) had no effect on the amount of bacteria transmitted, indicating that

bacteria persisted, and at least within 24 h did not noticeably proliferate, in the flies' salivary gland, digestive tract and/or on their mouthparts. Comparably, stable flies transmitted *Enterobacter sakazakii* – a bacterium causing necrotizing enterocolitis, sepsis, and meningitis – as many as 20 days after they had ingested it (Mramba et al. 2007).

Our laboratory data clearly show that stable flies are attracted to *S. aureus*, and are capable of transmitting *S. aureus* between sources, with the rate of transmission being dependent upon the amount of bacteria ingested by flies. In addition to oral bacterial transmission, bacteria may also be transmitted through physical contact when flies reside on cows while taking a blood meal. Regardless of the mode of bacterial transmission, attraction of stable flies to *S. aureus* should also be demonstrated in field settings. Such studies, however, raise safety concerns because *S. aureus* is a Risk Group 2 microbe which requires special permits for field testing from the Government of Canada. Instead of field-testing *S. aureus* itself, its semichemical gases and odorants could be identified and presented as a trap lure for fly attraction. To investigate whether wild stable flies carry *S. aureus*, flies could be field-collected around dairy farms and tested for the presence of the bacteria, analogous to studies that tested wild stable flies for the presence of the *Anaplasma marginale* bacteria which cause anaplasmosis in cattle (Araujo et al. 2021), and for the presence of *Treponema* bacteria which cause digital dermatitis in dairy cattle (Thibodeaux et al. 2021). If correlations were found between the bacterial infection level of flies and the incidence of cattle mastitis, these correlations would support the concept of a positive feedback loop (Figure 4.5), where flies are attracted to mastitis-afflicted cows, worsen their infection, and spread *S. aureus* to healthy cows.

4.5) Acknowledgements

We thank Kevin Anderson for supplying the eight strains of *S. aureus* tested for stable fly attraction, Stephen Takács for help with Figure 4.1, and Adam Blake for help with statistical analyses and Figure 4.3. Figure 4.5 was created using BioRender.com. SN was supported by the Thelma Finlayson Graduate Entrance Scholarship, the Mutual Fire Insurance Company of British Columbia Graduate Scholarship, the Dr. John Yorston Memorial Graduate Scholarship in Biological Sciences, the John and Lyn Webster

Graduate Scholarship in Biological Sciences, and by a Simon Fraser University Graduate Fellowship. Further funding for this project was provided by a Natural Sciences and Engineering Research Council of Canada (NSERC) – Industrial Research Chair to GG, with BASF Canada Inc., and Scotts Canada Ltd. as the industrial sponsors.

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4.7) Figures

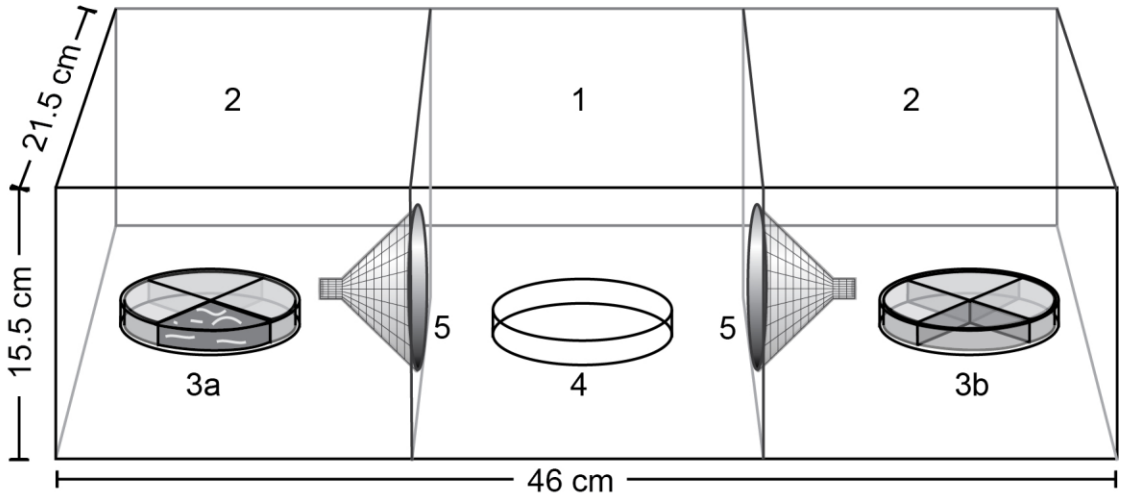

Figure 4.1 Three-chamber still-air olfactometer with a central chamber (1) and two lateral chambers (2). In each bioassay, the lateral treatment chamber was baited with a plate of three sterile agar slices, and one slice of agar growing a strain of *Staphylococcus aureus* or the proven attractive *S. sciuri* (Nayani et al., 2023a) (3a), whereas the lateral control chamber was baited with a plate of four sterile agar slices (3b). To initiate a replicate, 20 blood- and water-deprived female stable flies were released from a Petri dish (4) into the central chamber of the olfactometer and given 24 h to enter lateral chambers through mesh funnels (5).

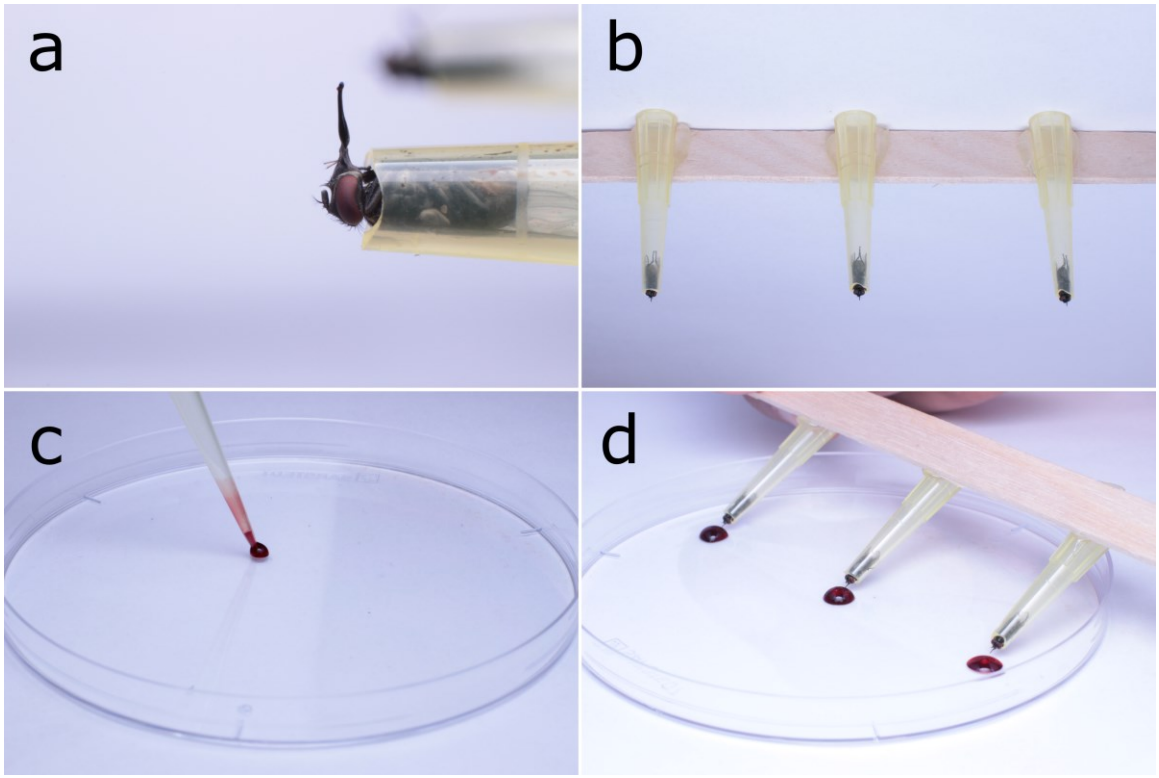

Figure 4.2 Photographs illustrating the protocol for blood-feeding stable flies. **(a)** Pipette-tip (200 µL) adapted as a 'fly restraint', with only the head and mouthparts of the fly protruding from the cut pipette tip. (**b**) Three fly restraints, each confining one fly, hot-glued 3.5 cm apart on a popsicle stick. (**c**) Droplet (20 µL each) being pipetted on a sterile Petri dish. (**d**) Three flies in separate restraints concurrently ingesting blood.

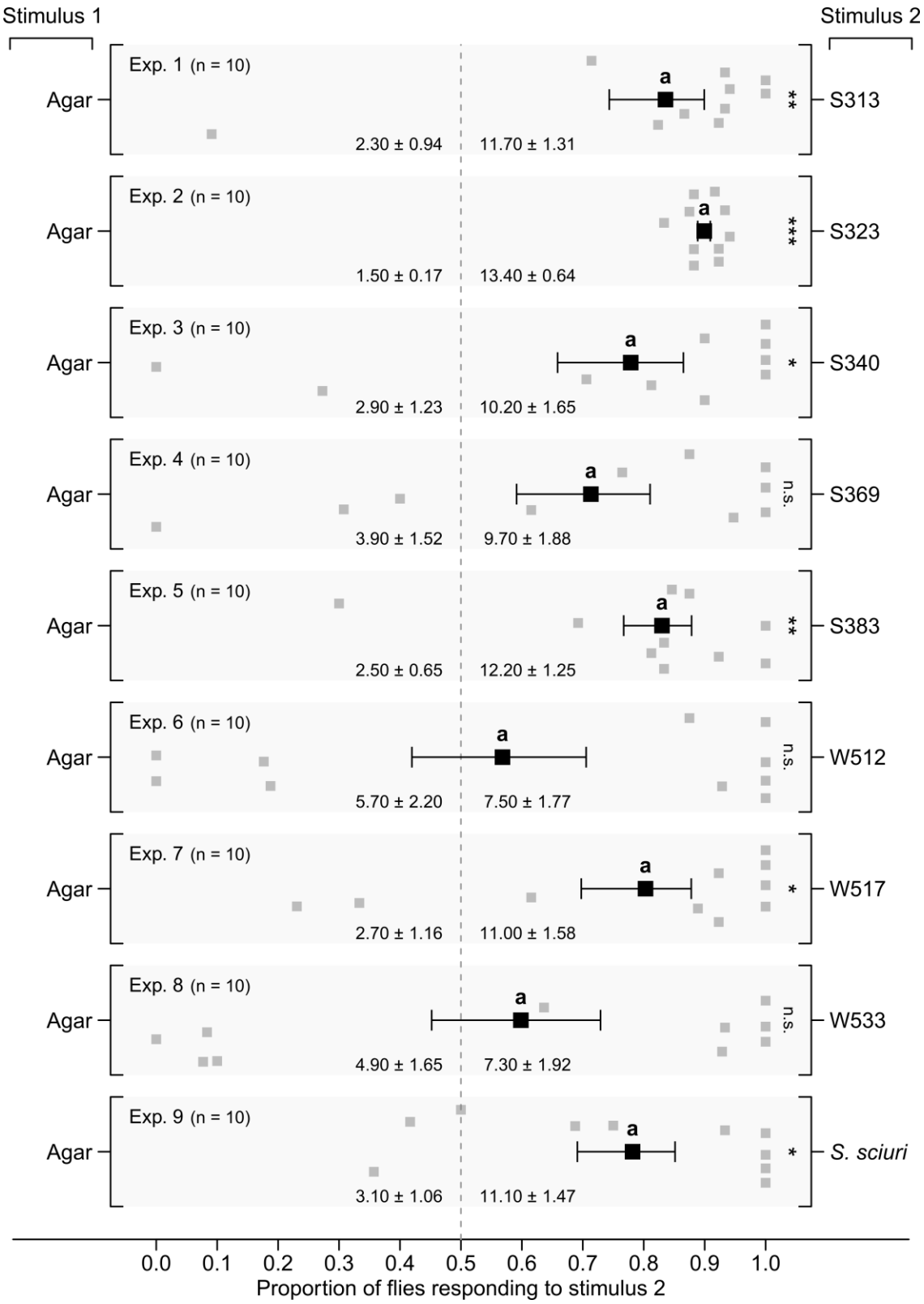
Stimulus 1
Stimulus 2
Exp. 1 (n = 10)
Agar
2.30 ± 0.94
11.70 ± 1.31
a
**
S313
Exp. 2 (n = 10)
Agar
1.50 ± 0.17
13.40 ± 0.64
a
***
S323
Exp. 3 (n = 10)
Agar
2.90 ± 1.23
10.20 ± 1.65
a
*
S340
Exp. 4 (n = 10)
Agar
3.90 ± 1.52
9.70 ± 1.88
a
n.s.
S369
Exp. 5 (n = 10)
Agar
2.50 ± 0.65
12.20 ± 1.25
a
**
S383
Exp. 6 (n = 10)
Agar
5.70 ± 2.20
7.50 ± 1.77
a
n.s.
W512
Exp. 7 (n = 10)
Agar
2.70 ± 1.16
11.00 ± 1.58
a
*
W517
Exp. 8 (n = 10)
Agar
4.90 ± 1.65
7.30 ± 1.92
a
n.s.
W533
Exp. 9 (n = 10)
Agar
3.10 ± 1.06
11.10 ± 1.47
a
*
S. sciuri
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
Proportion of flies responding to stimulus 2

Figure 4.3 Back-transformed estimated marginal mean (± SE) proportions of female stable flies captured in lateral chambers of still-air olfactometers (Figure 4.1). Control chambers were baited with a plate of four sterile agar slices (Stimulus 1), whereas treatment chambers were baited with a plate of three sterile agar slices, and one slice of agar growing a strain of *Staphylococcus aureus* (S313, S323, S340, S369, S383, W512, W517, W533; Exps. 1-8), or the proven attractive *S. sciuri* (Nayani et al., 2023a) (Exp. 9) (Stimulus 2). For each experimental replicate, 20 blood- and water-deprived female flies were released into the central chamber of the olfactometer and given 24 h to enter lateral chambers. Grey symbols show the proportion of flies captured in individual replicates in response to Stimulus 2, whereas the black symbol shows the mean. Mean numbers of flies captured in response to stimuli in experiments 1-9 are listed at the bottom of each graph; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, as determined by a likelihood ratio test; mean proportions in different experiments labelled with the same letter do not differ statistically, post-hoc Tukey tests, $P > 0.05$.

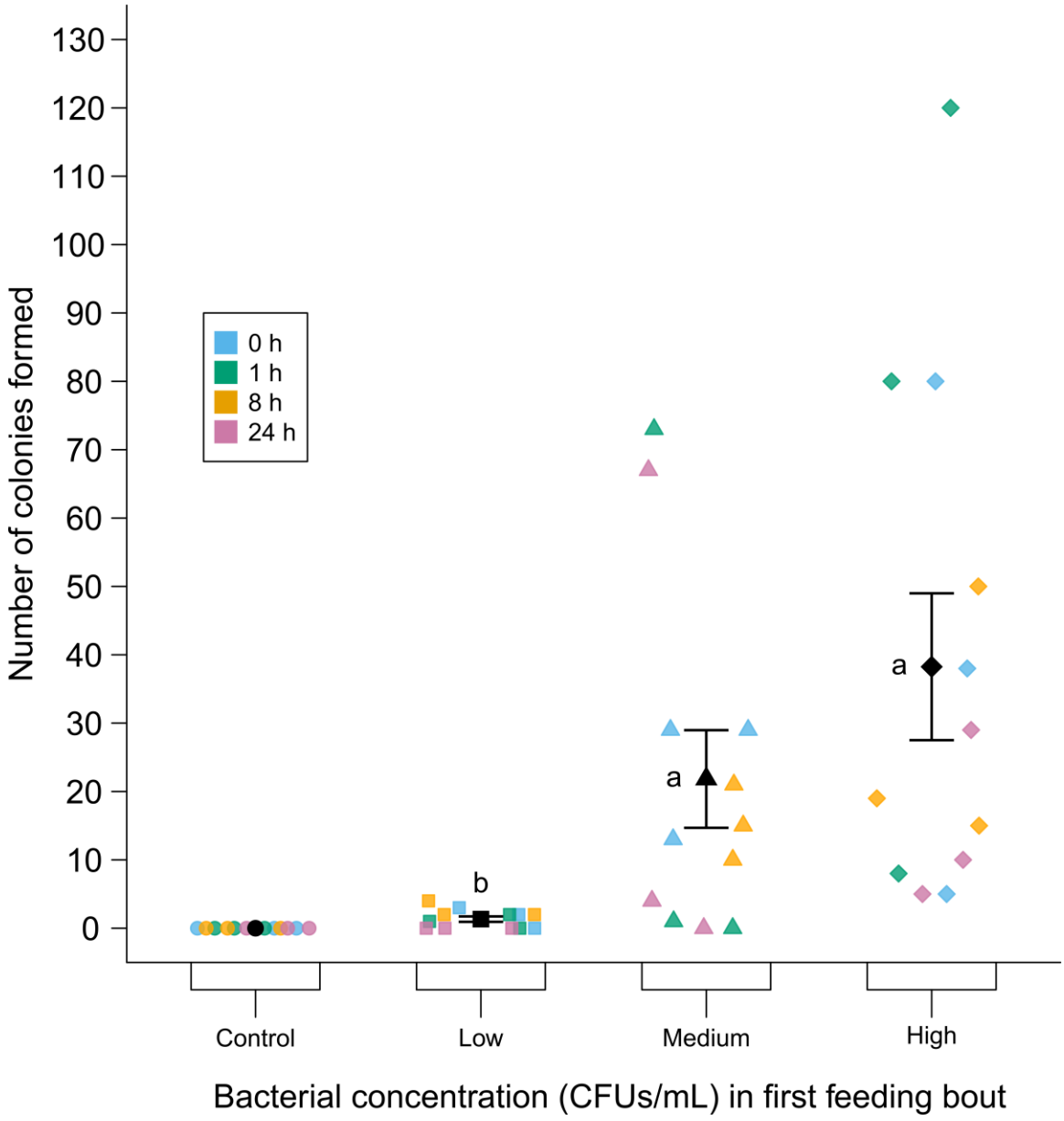

Figure 4.4 Back-transformed estimated marginal mean (± SE) numbers of *Staphylococcus aureus* colonies that formed when plating sterile blood after being fed upon by female stable flies. In their first feeding bout, flies ingested droplets of blood inoculated with *S. aureus* at doses of 0 (control), 10^5 (low), 10^7 (medium) and $>10^9$ (high) colony forming units per milliliter (CFUs/mL); in their second feeding bout, flies ingested sterile blood, transmitting *S. aureus* in the process. The dose of *S. aureus* in blood droplets (zero/control, low, medium, high) fed upon by flies during their first feeding bout dose-dependently affected the number of CFUs that flies transmitted to

sterile blood during their second feeding bout ($\chi^2 = 18.06$, df = 2, $p < 0.05$) but the time elapsed between the first and second feeding bout (0 h, 1 h, 8 h and 24 h) had no effect on the amount of bacteria transmitted to sterile blood. Mean numbers of colonies formed between treatment doses labelled with different letters differ statistically (Tukey adjusted, $P < 0.05$).

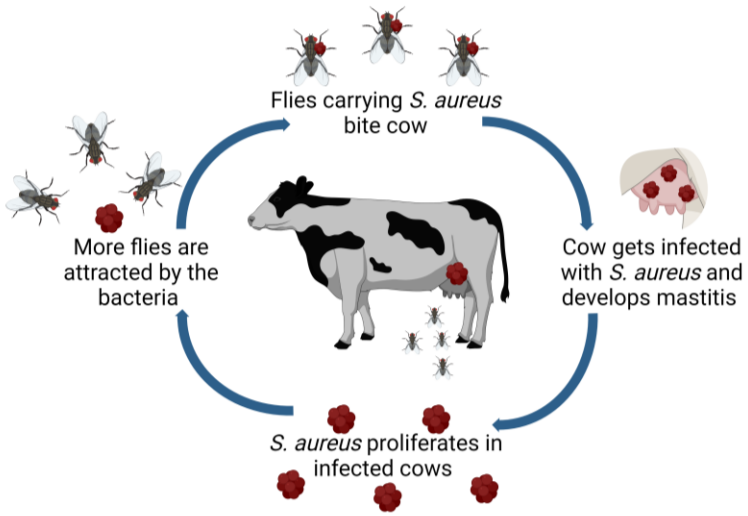

Figure 4.5 Proposed positive feedback loop depicting the process of a healthy cow becoming infected, and reinfected, with *Staphylococcus aureus*, thus causing bovine mastitis. When stable flies carrying *S. aureus* bite a cow, they transmit *S. aureus* to that cow, thereby causing an infection and the development of mastitis. Proliferating bacteria then attract even more flies and worsen the infection. Flies attracted to a heavily afflicted cow ingest a large dose of *S. aureus* which enables them to transmit *S. aureus* to healthy cows.