Foraging and Communication Ecology of the Common Green Bottle Fly, *Lucilia sericata* (Meigen) (Diptera: Calliphoridae)

by

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Abstract

In accordance with their physiological state, adults of *Lucilia sericata* must locate mates, food and oviposition resources. I investigated the cues they exploit to obtain these resources.

As females require a protein-rich diet and frequently visit pollen/protein-rich flowers, I studied the effects of generic floral scent and colour cues, and of Oxeye daisy-specific cues, on foraging decisions by flies. I show that (1) flies in the presence of generic floral scent respond more strongly to a uniformly yellow cue than to most other uniform colour cues (green, white, black, blue, red); (2) daisy scent enhances the attractiveness of a yellow cue; and (3) pollen with adequate moisture content facilitates oocyte maturation of flies.

Males respond to long-range mate recognition cues. I show that (1) wing movement of females is a visual mate recognition cue, (2) wings are thin-film reflectors that produce light flashes during movement, and (3) light flashes are absent under diffuse light. Wings also produce stable structural colours, UV- and polarized-light reflections, but these optic effects *per se* are insufficiently gender-specific and thus do not appear to serve as mate recognition cues. Instead, the frequency of light flashes reflected off moving female wings may allow males to recognize prospective mates.

Foraging decisions by females change in accordance with their physiological state. Protein-hungry females respond to feces and carrion, whereas protein-fed gravid females with mature oocytes respond only to fresh carrion. Gravid females discriminate against aging carrion (which is detrimental to their offspring) as soon as it produces appreciable amounts of indole, which is an abundant feces semiochemical and apparently serves as an indicator of a food rather than an oviposition resource.

Gravid females locate recently deceased vertebrates as oviposition sites in response to dimethyl trisulfide and carrion-type colour cues (dark red, black), indicating that a bimodal cue complex signifies suitable oviposition sites.

Oviposition site-seeking females do not respond to an oviposition pheromone. Instead, they coopt semiochemicals associated with feeding flies as resource indicators. This conclusion is based on data that gravid or non-gravid females ovipositing and/or feeding on oviposition resources enhance their attractiveness to gravid and non-gravid females.

Keywords: *Lucilia sericata*; Calliphoridae; Multimodal communication; Physiology- and development- based resource location; Vision and olfaction; Resource semiochemicals

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List of Acronyms

Term	Initial components of the term
DMTS	Dimethyl trisulfide
GC MS	Gas chromatography-mass spectrometry
GCEAD	Gas chromatographic-electroantennographic detection
HSV	Head space volatiles
RF	Response fly
SB	Synthetic blend
SF	Stimulus fly
Term	Initial components of the term
UV	Ultra violet
WIP	Wing interference pattern

Glossary

Term	Definition
Aggregation	A group of individuals at one location
Aggregation pheromone	Intraspecific chemical signal that induces group formation (Wertheim et al. 2005)
Behaviour	The way in which an organism adjusts and interacts with its environment (Matthews & Matthews, 1978)
Contact pheromone	Chemicals sensed upon physical contact
Cue	A stimulus, animate or inanimate, that elicits a type of behaviour (Hasson, 1994; Maynard, Seely, 1989; Smith & Harper, 2003)
Fitness	Genetic contribution to future generations.
Kairomone	Chemical cue that mediates interspecific interaction, benefiting only the receiver
Multimodal communication	Transfer of information to con- or hetero-specifics using signals of different sensory modalities (Higham & Hebets, 2013)
Olfactory	Relating to odour
Oviposition pheromone	Intraspecific semiochemical signal that induces oviposition by conspecifics
Pheromone	Intraspecific chemical signal that induces a behavioural or physical response typically benefiting both the signaller and the receiver
Semiochemical	Message-bearing chemical
Signal	Means of intentional information transfer
Visual	Relating to sight

Chapter 1.

Introduction

In the introductory chapter of my thesis, I will introduce the main field of my research, present rationale for studying blow flies, describe the general biology of blow flies, and point out research opportunities. I will also provide a brief summary of each research chapter, highlighting key results.

1.1. Background information

Multi-sensory or multi-modal integration of information is widespread in the animal kingdom. Most animals, including insects, respond to combinations of visual, chemical, acoustic, tactile, and thermal signals or cues. Multimodal information transfer refers to instances where each type of signal or cue on its own may elicit a behavioural response but where there is generally complex interaction between two or more sensory modalities (Higham and Hebets 2013). The information transfer can either be intentional (signal) or unintentional (cue) but has evolved for rapid and accurate sensing and recognition of the signal or cue within complex and "noisy" environments.

Orientation of insects towards a resource is thought to be a hierarchical process where the insect relies on different sensory modalities dependent upon its distance from the resource. Long-range mate location is mediated by various types of signals including pheromones [as in many Lepidoptera (Hansson 1995; Wei et al. 2006; Loriatti et al. 2011), Hemiptera (Aldrich 1988; Moraes et al. 2008; Dewhirst et al. 2010), Hymenoptera (Ayasse et al. 2001; Crook et al. 2012), Coleoptera [Allison et al. 2004; Gitau et al. 2013; Vuts et al. 2014), and Diptera (Wicker-Thomas 2007)], sounds [as commonly in the Orthoptera (Robinson and Hall 2002)], and colour displays [as often in diurnal Lepidoptera (Sivinski and Wing 2004; Kemp et al. 2005), Coleoptera (Lloyd 1971) and

Diptera (Allan et al. 1987; Yuval 2006)]. As an insect then approaches a prospective mate, it gathers short(er)-range information often with a different sensory modality to pinpoint the micro-location of the signalling mate, as shown in buprestid beetles (Coleoptera: Buprestidae) (Pureswaran and Poland 2009), vespid wasps (Hymenoptera: Vespidae) (Tibbetts 2004), and pentomatid shield and stink bugs (Hemiptera: Pentatomidae) (Borges et al. 2011). Upon alighting on or near the prospective mate, mate assessment proceeds in response to contact pheromones (Blomquist and Bagnéres 2010)], visual characteristics (Kemp et al. 2011), body size (Bonduriansky 2001)], and tactile and vibratory information (Čokl et al. 1999; Uzsák 2014)]. Mate location by the green stink bug, *Nezara viridula* (L.) (Hemiptera: Pentomatidae), exemplifies this hierarchical process, involving a long-range pheromone (Brézot et al. 1994) and short(er)-range sound (Miklas et al. 2003), vibratory (Čokl et al. 1999), and visual signals (Borges et al. 1987). The concept of long- and short-range information transfer also applies to foraging cues. For example, foraging females of the yellow fever mosquito, *Aedes aegypti* (L.) (Diptera: Culicidae) (van Breugel et al., 2015), use a hierarchy of three sensory cues when they locate a blood host. At long range, they respond to a plume of CO_2 from the host before they narrow in at close range on host-related visual and thermal cues, possibly also guided by host odour cues that may help them choose one host over another (van Breugel et al. 2015).

The hierarchical process of locating a resource, or the preference for specific resources, may change dependent upon the type of resource (e.g., mate, food, oviposition site), and the ontogenetic development or physiological state of the resource-seeking insects that typically need multiple resources throughout the course of their life. For example, many emergent female mosquitoes seek nectar resources for energy (Smith and Gadawski 1994, Gary and Foster 2006), then blood hosts to mature their oocytes (Foster and Takken 2004; Qiu et al. 2011; Verhulst et al. 2011), and finally suitable sites to oviposit (Osgood 1971; Olagbemiro et al. 2004; Navarro-Silva et al. 2009; Barbosa et al. 2010), possibly repeating one or more of these tasks.

For my thesis I chose to study the foraging and communication ecology of blow flies. I selected blow flies as study organisms because they have an intriguing biology and ecology, and they offer fascinating research opportunities that I describe in the next

section. I focused on the common green bottle fly, *Lucilia sericata* (Meigen) (Diptera: Calliphoridae), because it is a representative blow fly species in the northern hemisphere (Hall 1948, Hall and Townsend, 1977) and an early responder to animal carrion (Davis 1928; Cragg and Hobart 1955; Cragg 1956; Hall and Doisy 1993; Byrd and Castner 2010).

1.2. General biology of calliphorid flies and research opportunities

Blow flies are holometabolous insects with egg, larval, pupal, and adult stages. An adult gravid female blow fly lays approximately 200 eggs in a single clutch and may lay multiple clutches throughout her life. The eggs are pale yellow and resemble small pieces of rice (1.5 mm x 0.4 mm). In most calliphorid species, a 1:1 sex ratio of offspring prevails but females of *Chysomia rufifacies* (Macquart) (Ullerich 1973) and *C. albiceps* (Wiedemann) (Schnack and Muzon 1995) can produce only sons or daughters. From eggs deposited on carrion, larvae (maggots) hatch within 8-12 (Byrd and Castner 2001) hours and selectively consume certain portions of the resource. Through proteolytic enzymes in their saliva and excreta, and through mechanical grinding by mouth hooks, larvae break down and feed on resource proteins. Larvae develop through three instars within 5-15 days at 15-28 ºC (Higley et al. 2014) and then leave the carrion to pupate in nearby soil, forming puparia that range in colour and size (~ 10 mm) based on species and larval diet. The time period from egg to pupa varies considerably between species and is dependent on temperature.

Following a 10- to 20-day pupation period, the adult fly ecloses by pushing out of its puparium with an eversible bladder (ptilinum) between the eyes. After the adult fly has eclosed, the ptilinum deflates and is never used again. Adult male and female flies are approximately 6-12 mm long, and are morphologically distinct in that the compound eyes of females, but not males, are separated by a large gap. Eclosed blow flies must forage for food, seek mates, and locate oviposition resources. Below, I shall describe the challenges that blow flies face during their search for these resources, and outline the research opportunities that present themselves in these contexts.

1.2.1. Foraging for food

Foraging success determines the ability of insects to survive and reproduce (Webber 1956; Stone et al. 2009). Blow flies obviously forage for, and feed on, decomposing carcasses (Hayes et al. 1999; Archar and Elagar 2003), but they also obtain protein from animal feces, fungi, and urban dumpsters (Stoffolano et al. 1995). Blow flies even visit pollen-rich composite flowers and nectar-rich umbelliferous flowers (Karczewski 1967), suggesting that they feed on pollen protein as well as nectar, responding to specific floral cues.

It is well known that blow flies obtain carbohydrates from floral nectar (Grinfel'd 1955; Mullen and Durden 2009), and require carbohydrate meals before they feed on protein and seek mates (Smith and Gadawski 1994, Foster and Takken 2004, Gary and Foster 2006). However, whether blow flies are attracted to flowers, and the semiochemical and visual cues that may mediate attraction to flowers, are yet to be studied.

Blow fly females need a substantial amount of protein to mature their oocytes (Erzinclioglu 1996). Flower pollen is readily available on the composite flowers that flies visit. Pollen ranges from 2.5% to 61% in protein content (Roulston et al. 2000) and consequently may be a better protein resource than animal feces, which ranges from 0.5% to 7% in protein content and which is known to be a poor diet for rapid egg maturation (Clift and McDonald 1976; Readshaw and Vangerwen 1983; Stoffolano et al. 1995; Wardhaugh et al. 2008). However, whether or not blow flies can consume, digest, and ultimately supplement their diet with pollen protein has not yet been reported.

Although blow flies feed on carrion at advanced stages of decay, gravid flies reject the same resource for oviposition (Archer and Edgar 2003; Huntington 2008), implying both an effect of physiological state on the flies' resource preference, and changing semiochemical cues associated with an aging carrion resource. The effect of physiological state on foraging decisions by adult blow flies, and the foraging cues that they exploit to gauge the decomposition state of a carrion resource, have yet to be reported.

1.2.2. Seeking mates

Blow flies are reported not to have sex pheromones or sexually dimorphic cuticular hydrocarbons (Stoffolano et al. 1997), but to be highly visual (Hardie et al., 2012), and hilltop (waiting on a location of high elevation to recognize females; Merz 2000), , suggesting that blow flies may rely on vision as the dominant sensory modality to locate mates. Necrophagous male flies that locate females within their territory (Land and Collet 1975; Moore 2014; B. Brodie, pers. obs.) engage in a fast-paced courtship chase (Erzincliogl u 1996; Boeddeker et al. 2003), implying the involvement of visual cues in mate location and pursuit.

Blow flies respond to visual cues (Wall and Fisher 2001; Boeddeker and Egelhaaf 2003; Gomes et al. 2007) and possess eyes capable of detecting ultra violet (UV) and polarized light (Meffert and Smola 1976; Smola and Meffert 1979; Briscoe and Chittka 2001). It follows that stable structural colours in blow fly wings (= wing interference patterns), and UV- and polarized-light reflections off the flies' wings or integument, may represent cues that help blow flies locate mates. This prediction has yet to be experimentally tested.

1.2.3. Locating oviposition sites

Vertebrate carcasses are fleeting resources that are limited in time and space. Throughout the decomposition process, they are exploited by scavenging terrestrial vertebrates such as insects, fungi, and microbes (DeVault et al. 2003). Blow flies may avoid inter-specific resource competition by responding immediately (Anderson and VanLaerhoven 1996) or within hours (Hall and Doisy 1993; Mullen and Durden 2009) to a recently deceased animal, sensing decomposition semiochemicals such as dimethyl trisulfide (DMTS) (Nilssen et al. 1996). Blow flies appear on carrion long before it becomes repulsive to the human nose. Thus, kairomones other than sulfides, or other highly volatile components, may disseminate from the corpse and attract flies. Alternatively, flies may be able to detect and respond to very low quantities of sulfur-containing semiochemicals that are not detectable by the human nose. Regardless, the essential semiochemical cues that attract gravid blow fly females to a recently deceased animal are yet to be identified.

Ovipositing females enhance the attractiveness of a carrion resource directly proportional to the number of flies engaged in oviposition (White 2006). Females of the Australian sheep blow fly, *Lucilia cuprina* (Wiedemann), are stimulated to oviposit by tactile and pheromonal signals from conspecifics (Browne et al. 1969). If the reproductive biology of blow flies is resource-dependent or -related, then oviposition pheromones may attract both males and females. Females may be attracted to protein resources for feeding or oviposition, whereas males may be attracted to the same sites to locate mates. If oviposition pheromones indeed modulate the attractiveness of oviposition sites (Barton Brown et al. 1969; Ashworth and Wall, 1994; Wertheim et al. 2005), then these pheromones are yet to be identified.

1.3. Chapter summaries

My thesis consists of 7 chapters. The first chapter is a brief introduction of my study area, followed by five research chapters, and a concluding chapter summarizing my major findings. The thesis is organized in an article-style, with thesis chapters closely resembling manuscripts that have already been published (chapters 5 and 6), have been submitted for peer review (chapters 2 and 3), or are in preparation (chapter 4). Each chapter is presented in the format that is required by the journal where the corresponding manuscript has been, or will be, submitted for review. Each research chapter includes an abstract, introduction, methods, results, discussion, and a reference list. The following is a brief outline of each chapter.

In chapter 2, I study the effects of generic floral scent and colour cues, and of Oxeye daisy-specific cues, on foraging decisions by *L. sericata*. In laboratory experiments, I show that (*i*) flies in the presence of a generic floral scent respond more strongly to a uniformly yellow cue than to most other uniform colour cues (green, white, black, blue, red), (*ii*) daisy scent enhances the attractiveness of a yellow cue, and (*iii*) pollen with adequate moisture content facilitates oocyte maturation of flies. With evidence that *L. sericata* exploits floral cues during foraging, and that pollen nutrients can serve as an alternate or supplement to animal feces nutrients, I conclude that pollen may play a major role in the foraging ecology of *L. sericata* and possibly other filth flies. I further conclude that blow flies may play a significant role as pollinators.

In chapter 3, I demonstrate that foraging decisions by *L. sericata* females change in accordance with their physiological state. Specifically, I show that protein-hungry females respond to feces and carrion, whereas protein-fed gravid females with mature oocytes respond only to fresh carrion. With DMTS shown to attract gravid *L. sericata* females to carrion (see Chapter 5), I focused my study on identifying headspace volatiles from canine feces. I show that a blend of indole and one or more of the alcohols phenol, *m-/p*-cresol and 1-octen-3-ol is as attractive to *L. sericata* females as canine feces. In laboratory and field experiments, I further study how gravid females distinguish between fresh and aging oviposition resources, the latter being possibly detrimental to their offspring. I show that gravid females appear to accomplish this task, in part, by responding to trace amounts of DMTS that emanate from fresh carrion and by discriminating against carrion as soon it begins to produce appreciable amounts of indole, which is the second most abundant semiochemical in fresh canine feces. I conclude that indole may serve as an indicator of a food rather than an oviposition resource.

In chapter 4, I study mate recognition in *L. sericata.* I present data demonstrating that males respond to long-range visual, rather than semiochemical, mate-recognition cues. I show that (1) wing movement of females is a mate recognition cue, (2) wings are thin-film reflectors that produce light flashes during movement, and (3) that light flashes are absent under diffuse light. I present evidence that wings also produce stable structural colours, UV- and polarized-light reflections, but that these optic effects *per se* are insufficiently gender-specific and thus do not appear to serve as mate recognition cues. Instead, the light flashes reflected off moving female wings seem to allow males to recognize prospective mates.

In chapter 5, I investigate the semiochemical and visual cues that mediate attraction of gravid *L. sericata* females to fresh rat carrion. I show that female flies are more strongly attracted to incised rat carrion than to intact carrion, and that the attraction is mediated entirely by DMTS. In both laboratory and field experiments, I show that increasing concentrations of DMTS attract increasingly more flies. When I coupled DMTS with specific visual cues, I could demonstrate that carrion-type colour cues (dark red, black) are more effective than bright colour cues (white, yellow) in attracting flies.

When I baited dark traps with DMTS in field experiments, I captured a total of 214 calliphorid flies (200 *L. sericata)*, all of which were gravid females. These results support my conclusion that DMTS and dark colour represent a bimodal cue complex that signifies suitable oviposition sites to gravid calliphorid females, particularly *L. sericata*.

In chapter 6, I study the underlying mechanisms of aggregated oviposition by *L. sericata* and *Phormia regina* (Meigen) (Diptera: Calliphoridae). I provide evidence in support of the conclusion that oviposition site-seeking *L. sericata* females do not respond to an oviposition pheromone. Instead, they coopt semiochemicals associated with feeding flies as resource indicators, taking chances that resources are suitable for oviposition, and that ovipositing flies are present. This conclusion is based on data that gravid or non-gravid females ovipositing and/or feeding on oviposition resources enhance their attractiveness to gravid and non-gravid females.

In chapter 7, I summarize my major findings and highlight their implications.

1.4. References

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Chapter 2.

Effects of floral scent, colour and pollen on foraging decisions and ovary development of common green bottle flies

Brodie, B.S., M. Smith, J. Lawrence, and G. Gries. A very similar (manuscript) version of this Chapter is in peer review – (PloS One)

2.1. Abstract

The common green bottle fly *Lucilia sericata* and other filth flies frequently visit pollen-rich composite flowers such as the Oxeye daisy, *Leucanthemum vulgare*. In laboratory experiments with *L. sericata*, we investigated the effect of generic floral scent and colour cues, and of Oxeye daisy-specific cues, on foraging decisions by recently eclosed flies. We also tested the effect of a floral pollen diet with 0-35% moisture content on the ability of females to mature their oocytes. Our data indicate that (1) young flies (1-3 days old) in the presence of generic floral scent respond more strongly to a uniformly yellow cue than to any other uniform colour cue (green, white, black, blue, red) except for ultraviolet (UV); (2) the floral scent of Oxeye daisies enhances the attractiveness of a yellow cue; and (3) moisture-rich pollen provides nutrients that facilitate ovary maturation of flies. With evidence that *L. sericata* exploits floral cues during foraging, and that pollen can be an alternate protein source to animal feces and carrion for nutrition. Pollen apparently plays a major role in the foraging ecology of *L. sericata* and possibly other filth flies. These flies, in turn, may play a significant role as pollinators, as supported by a recently published study.

2.2. Introduction

Flies (Diptera) are the second most important group of flower-visiting insects world wide (1). Flies particularly in the sub-orders *Nematocera* and *Brachycera* are well known to visit flowers in search for nectar and pollen (1,2). The common green bottle fly, *Lucilia sericata* (Meigen) (Diptera: Calliphoridae), and other calliphorid blow flies are well known to feed on carrion and feces but are also frequently observed on plant inflorescences (3-6). Yet, the foraging cues they exploit to locate pollen- or nectar-producing plants, and the potential nutritional benefits of pollen and nectar on ovary development and maturation in flies have not yet been investigated.

To mature their oocytes, blow flies require large amounts of protein (7-9) which they obtain from various resources including carrion and animal feces (10,11). Although of high quality, carrion protein is ephemeral and thus less dependable as a nutritional resource (12,13). In comparison, animal feces has a low protein content (0.5-7%) (10,11,14-16) and, as such, represents a poor diet for the rapid maturation of fly oocytes prior to oviposition. Flower pollen, in contrast, has a protein content of 7% to 80% and contains low-molecular-weight proteins (17), which are essential for oocyte development (18,19). Thus, pollen, as a resource, may be more reliable and possibly more suitable than animal feces.

Frequent protein meals are crucial for gravid blow fly females to develop and maintain their reproductively mature oocytes, which they otherwise resorb (16,20). When protein-deprived females of the flesh fly *Sarcophaga bullata* Parker (Diptera Sarcophagidae) are given a food choice between an amino acid-sugar mix and sugar, they feed on the former suggesting that these females detect the amino acids and exhibit a nutrition-based feeding preference (21,22). In addition to the nutritional value of amino acids and proteins, flies require carbohydrates throughout their lives to fuel their daily activities and to increase their fecundity (23). For example, carbohydrate-deprived females of the mosquito *Anopheles gambiae* Giles (Diptera: Culicidae) produce fewer

and smaller eggs than well-fed mosquitoes (24). Evidence that blow flies can digest both pollen protein as well as floral nectar supports the two concepts. That blow flies visit plant inflorescences to obtain food and that they may become obligate foragers on floral pollen and nectar when animal feces and carrion are scarce. Expectedly then, calyptrate flies (like blow flies) are main pollinators in agricultural systems and are effective pollinators of some plant species (25,26).

Fly pollinators respond to both olfactory and visual cues of plant inflorescences (6,27). The attractiveness of inflorescences, though, seems to depend on the flies' reproductive status and physiology. Inflorescences of sapromyophilous plants such as the dead horse arum lily, *Helicodiceros muscivorus* (Schott ex. K. Koch), often appear dark red and emit decomposition odourants including dimethyl disulfide and DMTS. These odourants are reminiscent of carrion odour (28,29), and thus are particularly attractive to gravid female blow flies seeking oviposition sites (30). Unlike sapromyophilous flowers, myophilous flowers reward visitors with nectar, and possibly pollen, and produce a broad range of colours and typically sweet smelling fragrances (31). The fragrances include monoterpenes, fatty acid-derived acids, alcohols, and nitrogen-containing compounds (27,32) but lack the distinct oligosulfide-dominated stench of sapromyophilous flowers (33).

Pollen- or nectar-foraging blow flies may exploit both the semiochemical and visual inflorescence cues that the myophilous flowers have to offer. While both *L. sericata* and the Australian sheep blow fly *Lucillia cuprina* (Wiedemann) (Diptera: Calliphoridae) seem to have an innate affinity for yellow colours (34-36), it remains unknown whether they respond to yellow when they forage for nectar or pollen. It also remains unknown whether visual and semiochemical floral cues have interactive or synergistic effects on foraging decisions by blow flies. Such interactions are conceivable given that gravid *L. sericata* females respond well to a bimodal cue complex of dimethyl trisulfide and dark colour, two cues which in combination apparently signify suitable oviposition sites such as fresh carrion (30,37-40).

For our study we selected *L. sericata* because it is (*i*) commonly observed on flowers, (*ii*) frequently tested in the context of alternative pollination (5,6,41,42), (*iii*)

geographically wide-spread, (*iv*) representative of other flies within the family Calliphoridae (43,44), and (*v*) easily reared in the laboratory. As a model flower, we selected the Oxeye daisy, *Leucanthemum vulgare* Lam.*,* because it offers a large pollen disc as a protein source for pollinators, and it is commonly visited by blow flies.

We undertook this study to understand the effect of floral colour and odour on foraging decisions by *L. sericata* females, and the effect of pollen protein on the ability of females to mature their oocytes. We wanted to study whether pollen could possibly substitute animal feces as a protein source which, if so shown, would enhance the role of pollen as a food source for flies, and the role of flies as pollinators for composite flowers. Our specific research objectives were to investigate (1) the effect of floral colour cues on attraction of flies*;* (2) the effect of floral odour on attraction of flies; (3) potential interactions between visual and olfactory cues on attraction of flies; and (4) the effect of pollen on the ability of females to mature their oocytes.

2.3. Materials and Methods

2.3.1. Source of experimental flies and flowers

We reared flies in the insectary of Simon Fraser University (SFU), starting a new colony with approximately 50 gravid wild type flies every 12 months, and increasing the colony to about 5000 flies at specific times depending on experimental requirements. We kept flies under a L16:D8 photoperiod, at 30-40% relative humidity, and a temperature of 23-25 °C, providing water only, with the exception of Objective 4 in which flies were provided with a variety of food resources. We collected Oxeye daisies on SFU campus where they are abundant from mid-April to June, facilitating their deployment in various experiments.

2.3.2. General design of behavioural experiments

We tested the response of flies to visual and olfactory stimuli in 2-choice experiments 1-11 (Table 1), using BioQuip® (Compton, CA) wire mesh cages (61 × 61 × 61 cm) with a plated grey base (BioQuip®, Compton, CA, USA). Each cage was

illuminated from above with fluorescent lights (Phillips F32TA, Amsterdam, The Netherlands; light intensity in cage: 236 Lux). For each experimental replicate, we introduced 100 recently eclosed (1- to 3-day-old) cold-sedated flies of mixed sex (approximate sex ratio 1:1) into a cage, allowing them to acclimate for 2 h prior to the start of the experiment. We tested stimuli as part of an inverted "bottle trap" consisting of a 500-mL plastic Coke© bottle with the inverted top providing a cone-shaped funnel (11.5 cm long × 0.6 cm bottom diameter × 7.9 cm top diameter; Fig. 1A) that rested on the bottom half (20 cm long × 7 cm diameter; Fig. 1A), randomly assigning test stimuli to opposite corners of the bioassay cage. We covered the funnel with construction paper (11.5 cm long × 0.6 cm diameter) of a specified colour as the visual test cue (Fig. 1D) and consistently wrapped the bottom half with green construction paper (Fig. 1A). A mote of Sparkleen™ (Fisher Scientific Co. Pittsburgh, PA, USA) and water (0.5:5) in the trap bottom drowned the flies that entered the trap. We scored trap captures after a 6-h experimental duration.

Objective 1 (experiments 1-6): Investigate the effect of colour cues on attraction of *L. sericata*

To test whether flower-foraging flies may respond best to a bimodal complex of odour and visual (colour) cues, but to nevertheless isolate the effect of colour cues, we standardized the odour cue by using 2 g of honey (concentrated nectar) (Wedderspoon Organic Inc., Dancan, B.C., Canada) as a generic floral odour source that we placed in a 20-mL vial suspended by wire inside the inverted bottle traps (Fig. 1A). Honey odourants emanated from the vial through a mesh-covered hole (1.5-cm diameter) in the lid that prevented flies from accessing the honey, which is attractive to young *L. sericata* (Brodie, unpubl. data). We also standardized the presentation of visual test colour cues by shaping the construction paper such that it fit perfectly over the surface of the funnel-shaped trap top (Fig. 1B).

In each of parallel-run experiments 1-5 (Table 1), we tested the effect of a uniformly yellow cue *versus* that of a uniformly green, white, black, blue, or red cue [all colour cues: SunWorks® construction paper (Pacon Corporation, Appleton, WI, USA)]. Parallel-run experiment 6 (Table 1) had the same design but we deployed a different type of paper (Husky® copy30; Domtar, Montreal, QC, Canada) to generate ultra-violet

(UV) light reflections which are inflorescence cues known to attract bee and syrphid fly pollinators and to play a role as floral guides (45,46).

We measured the spectrometric profiles (*n* = 5 each) of all paper-derived colour cues and the spectrometric profiles of the daisies' petal tips, petal base, and floral disc using an Ocean Optics Inc. spectrometer (Dunedin, FL, USA).

Objective 2 (experiment 7): Investigate the effect of Oxeye daisy floral odour on attraction of *L. sericata*

Here we investigated whether not only generic honey odour, but also the specific inflorescence odour of Oxeye daisies, attracts *L. sericata* in the presence of a key visual cue (yellow) (Table 1). To this end, we fitted the funnel tops of each of the two traps with yellow construction paper and randomly assigned three freshly cut Oxeye daisy inflorescences with a 1-cm long stem each to one trap and three equivalent 1-cm long stem sections with the inflorescence severed to the other trap (Fig. 1C). We suspended plant material by piercing a wire through the plant tissue and attaching it to the funnel top of the trap (Fig. 1C).

Objective 3 (experiments 8-11): Investigate potential interactions between colour cues and Oxeye daisy inflorescence olfactory cues on attraction of *L. sericata*

In binary choice experiments 8-11 (Table 1), we tested for potential interactions between visual and olfactory cues on attraction of *L. sericata*. We generated floral odour cues by suspending three freshly cut Oxeye daisy inflorescences inside the trap (see above; Fig. 1C), and generated visual cues by covering the trap funnel with either yellow- or black-coloured construction paper (see above), while invariably covering each trap bottom with green-coloured construction paper. We selected yellow as a visual test cue because it is particularly attractive to young *L. sericata* females (see Results) that may be foraging for floral nectar and pollen (see below). We selected black because it is not very attractive to young females (see Results) but is part of a bi-modal cue complex (with DMTS) that is particularly attractive to gravid *L. sericata* females seeking oviposition sites (30). Using a factorial design, we specifically tested: (1) yellow with Oxeye daisy versus black with Oxeye daisy (Exp. 8); (2) yellow alone versus black alone (Exp. 9); (3) yellow alone versus black with Oxeye daisy (Exp. 10); and (4) yellow with

Oxeye daisy versus black alone (Exp. 11). To avoid carry-over effects of Oxeye daisy odour, we ran experiment 9 (which tested the effect of just visual cues) in a second bioassay room with identical temperature and relative humidity conditions as the first bioassay room. We ran experiments 9-11 with 2- and 3-day-old flies, with an equal number of replicates for each experiment on both days.

Objective 4 (experiments 12-17): Determine whether *L. sericata* females can mature their oocytes on a pollen diet

In Experiment 12-17 (Table 1), we tested the effect of a specific food source on the ability of *L. sericata* females to mature their oocytes, as follows: (1) granulated sugar (negative control); (2) Oxeye daisy pollen on 10 freshly cut inflorescences; (3-5) honey bee-collected pollen (The Honey Bee Centre, Surrey, BC, Canada) with a moisture content of 0% (3), 20% (4) and 35% (5); and (6) milk powder (positive control). For treatment 2, we collected Oxeye daisies on SFU campus and replaced them every day before the onset of the photophase. To produce the three levels of pollen moisture content, we placed honey bee-collected pollen in a desiccator and weighed it daily until there was no further weight reduction. We then finely ground the dry pollen for 5 min in a coffee grinder (Black and Decker; Towson, Maryland, USA) after which we added distilled water to produce pollen with 20-% or 35-% moisture content. As moisture content of pollen changes over time due to water evaporation, we replaced the pollen in treatments 3-5 every second day.

For each experiment, we placed 30 recently eclosed female flies (aged <1 day) in a wooden-framed cage with nylon mesh (25 cm high × 15 cm × 15 cm) and allowed them to feed on the food source *ad libitum* for 13 consecutive days. We then freeze-killed the flies and stored them in the freezer for later dissection. To assess the food effect on the maturation of oocytes**,** we removed the ovaries, placed them in a drop of Ringer's saline on a microscope slide, and scored 10 phases of follicle development according to the scheme of Adams & Reinecke (47) for the primary screwworm, *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae). As previously described (11), we also grouped phases of follicle development into three main stages: (I) phases 0-3: oocytes with dividing cells; (II) phases 4-9: oocytes with yolk sac, and (III) phase 10:

mature and chorionated eggs. In addition, we randomly selected flies to examine their ventriculus content for evidence of pollen.

2.3.3. Statistical analyses

We analyzed data of experiments 1-9 (objectives 1, 2: test for attraction of flies to colour (UV) cues and Oxeye daisy odour cues, respectively; see Table 1) with a non-parametric Wilcoxon signed rank test. For experiments 8-11 (objective 3: test for interactions between colour cues and Oxeye daisy odour cues on attraction of flies), we used generalized linear models with a Poisson distribution, corrected for overdispersion, and used the *number of fly captures* as the predicted variable and *odour*, *visual cue* and the interaction term *odour* × *visual cue* as the predictor variables (see Table 1). For each of experiments 1-11, we also used a chi-square goodness of fit test to analyze potential effects of floral colour, floral odour, or colour and odour interactions, on the sex ratio of responding flies. For experiments 12-18 (objective 4: test for effect of diet on fly ovary development), we used a logistic regression to test whether ovary development was dependent on diet. First, we used the *proportion of oocytes* at each of the three developmental stages (I, II, III) as the predicted variable, and *diet* (factor with 6 levels; see section 2.6.), *developmental stage* (factor with 3 levels), and the interaction term *diet* × *developmental stage* as predictor variables. Second, we ran separate logistic regression analyses for each developmental stage, using the *proportion of oocytes at that particular stage* as the predicted variable, and *diet* as the predictor variable. We then performed pairwise comparisons between diets to identify the effect of diet on ovary development using the Tukey HSD adjusted least square means test. We ran all statistical tests with JMP 10® (SAS Institute Inc.) for Windows® (Windows Corporation, Redmond, WA, USA).

2.4. Results

Objective 1 (experiments 1-6): Investigate the effect of colour cues on attraction of *L. sericata*

Yellow had a significant effect on captures of flies (Fig. 2; exps. 1-5). Traps with a yellow top captured significantly more flies than traps with a red top ($Z = 4.65$, $df = 1$, $p = < 0.0001$), blue top ($Z = 4.65$, $df = 1$, $p < 0.0001$), green top ($Z = 4.06$, $df = 1$, $p < 0.0001$), white top ($Z = 3.45$, $df = 1$, $p = 0.0006$) or black top ($Z = 4.48$, $df = 1$, $p < 0.0001$). In contrast, traps with a yellow top were not significantly more effective in capturing flies than traps with a UV light-reflecting top (Exp. 6: $Z = 0.00$, $df = 1$, $p = 1$). For each of experiments 1-6, there was no significant difference in the sex ratio of flies captured ($p > 0.05$).

Objective 2 (experiment 7): Investigate the effect of Oxeye daisy floral odour on attraction of *L. sericata*

Traps baited with Oxeye daisy inflorescences on 1-cm stems captured significantly more flies than traps baited with 1-cm stems alone (Fig. 3; $Z = -2.95$, $df = 1$, $p = 0.0028$). There was no significant difference in the sex ratio of flies captured ($p > 0.05$)

Objective 3 (experiments 8-11): Investigate potential interactions between colour cues and Oxeye daisy inflorescence olfactory cues on attraction of *L. sericata*

The colour of the trap top funnel (χ^2_1 (1, $N = 40$) = *22.83,* $p < 0.001$) and daisy odour (χ^2_1 (1, $N = 40$) = 22.8, $p = 0.003$) both had a significant effect on the number of flies captured (Fig. 4). Captures of flies increased in the presence of a yellow trap top or daisy odour, but there was no interaction between the colour yellow and daisy odour (Fig. 4.: χ^2_1 (1, $N = 40$) = 0.018, $p = 0.894$). For each of experiments 8-11, there was no significant difference in the sex ratio of flies captured ($p > 0.05$).

Objective 4 (experiments 12-17): Determine whether *L. sericata* females can mature their oocytes on a pollen diet

In randomly selected flies, we observed broken pollen grains in their gut. Females matured their oocytes to various degrees based on the type and moisture content of pollen. There was a significant interaction between food type and ovarian stage ($F_{17, 147}$ = 117.98, p <0.0001, Fig. 5). Stage-I oocytes were present in 33%, 28% and 22% of flies that had fed on sugar, Oxeye daisy pollen and 0% moisture content bee-collected pollen, respectively (Fig. 5). Stage-II oocytes were present in 22% of flies that had fed on bee-collected pollen with ≤20% moisture content. Lastly, stage-III oocytes were present in 48% and 39% of flies, respectively, that had fed on milk powder or bee-collected pollen with ≤35% moisture content (Fig. 5).

2.5. Discussion

Our study contributes to the current understanding of how and why blow flies forage for flowers, with *L. sericata* as the model fly species, and the Oxeye daisy as the model plant species among the composite flowers that blow flies so commonly visit (3-6). Our data indicate that (1) young flies respond more strongly to a uniformly yellow cue than to any other uniform colour cue (green, white, black, blue, red) except UV; (2) floral odour of Oxeye daisies enhances the attractiveness of yellow; and (3) pollen provides nutrients that contribute to ovary maturation of flies. Below we discuss the implications of our findings.

Of the many species of flies that are capable of colour vision (48-50), the Calliphoridae (blow flies), Syrphidae (hover flies), Tephritidae (fruit flies) and Anthomiidae (house flies) innately prefer yellow (35, 51-54). Our data corroborate the results of previous reports that blow flies prefer yellow, at least when flies are young and in flower-foraging mode. As expected, flower-foraging *L. sericata* females also respond well to UV, likely because the inflorescences of their commonly visited plants in the Apiaceae and Asteraceae often provide UV floral guides (45,55). That uniformly blue or red colours were least attractive to flies (Fig. 2; experiments 4, 5) may be attributed to evidence that these colours attract other pollinators and thus help partition floral

resources among floral visitors (51,56,57) that compete for nectar and pollen. Indeed, bumblebees primarily visit inflorescences with dark blue or violet (long) petals, whereas flies primarily visit inflorescences with yellow or white (short) petals. Expectedly then, plant species such *Raphanus raphanistrum* L. and *Myosotis sylvatica* Ehrh. (Family: Brassicaceae) with multiple floral colour morphs (58), or with floral colour changes over time (59), attract both fly and bee pollinators.

Floral odour alone or in conjunction with floral colour attracts insects (60,61) and plays a significant role in plant-pollinator interactions. Changes in floral odour alter its attractiveness to pollinators (62). For example, the omission of sulphur-containing odourants from floral odour mediate a shift from fly to wasp pollinator systems (63). Yet, blow flies are observed on the inflorescences of diverse plant species, many of which are not producing sulfur-containing or necromyophylous odourants (1,32,64). In our study, the sweat-like odour of Oxeye daisies clearly affected the behavior of flies (Fig. 3) but their responses were dependent upon the presence of a specific colour cue (Figs. 2, 4). Trap pairs with yellow or black funnel tops, each coupled with daisy odour, captured significantly more flies than the same trap pairs lacking daisy odour (Fig. 4). Similarly, when only one of the paired traps was coupled with daisy odour, the pair presenting a combination of yellow and daisy odour induced more fly captures than the pair presenting a combination of black and daisy odour (Fig. 4). The ineffectiveness of the latter combination could be explained by two factors: (1) *the mixed message that we deliberately presented*: naturally, daisy odour accompanies yellow rather than black floral cues. In contrast, carrion smell typically accompanies the dark pelt of the recently deceased animal. Simply put, there is no such thing as dark carrion smelling like daisy; and (2) *the physiological stage of flies altering their foraging response and preference (65, 66)*: recently eclosed flies need protein and carbohydrates to mature their oocytes (16,67) and respond well to a bi-modal cue complex of floral colour (e.g., yellow) and floral odour (e.g., daisy odour) that is indicative of pollen protein and nectar (this study). Gravid flies, in turn, that seek oviposition sites respond better to a cue complex of carrion odour and dark colour than to a complex of carrion odour and white or yellow colour (30). The above examples indicate not only that fly foraging decisions are affected by bi-modal cues more strongly than a mono-modal cue, they also indicate that combination preferences change in accordance with the physiological stage of the flies.

That flies respond to distinctively different and to bi- or multi-modal cue complexes when they forage for floral resources and oviposition sites, respectively, appears to be reflected in the type of cues that two sympatric plant congeners present to pollinators. The pale yellow and sweet-smelling inflorescences of *Metrodorea stipularis* Mart. likely appeal to protein- and nectar-foraging flies, whereas the violet-brown disagreeable-smelling inflorescences of *Metrodorea nigra* St. Hill. likely attract gravid flies (68).

Lucilia sericata females are indeed capable of digesting flower pollen. We observed broken pollen grains in the gut of flies and recorded at least partial maturation of oocytes in flies on a pollen diet (Fig. 5). This is noteworthy because few animals, including adult insects, can efficiently digest pollen (69). The pollen walls are apparently difficult to penetrate or dismantle. Insects gain access to the nutrient-rich cytoplasm in the center of pollen grains by inducing germination or pseudo-germination (70-72), bursting the pollen walls through osmotic shock (71), and by penetrating the pollen walls with digestive enzymes (69). Flies may pre-treat pollen with saliva for easier uptake or pre-digestion, and may pressure it with their proventriculus in the digestive tract that can break cell walls and thus facilitate access to the nutrient-rich cytoplasm. Given the importance of microorganisms to blow flies (74-75), it is conceivable that bacteria in the saliva and digestive tract of blow flies (76, 77) assist in the breakdown of pollen.

The ability of *L. sericata* females to digest pollen increased with the moisture content of pollen (Fig. 5), which averages 20% among plant species (78-79). The sponging mouthparts of flies may be more capable of handling moist pollen than dry pollen, and the former may be more easily processed through the digestive tract. Unexpectedly, our experimental flies could not mature their oocytes to stage III on Oxeye daisy pollen alone (Fig. 5). Advanced oocyte development may require specific pollen from multiple plants, which would explain why blow flies visit the inflorescences of many plant species (4-6), and why the bee-collected pollen with its diverse nutrient composition provided a diet suitable for ovary development to stage III (Fig. 5).

In summary, our data support the conclusion that flower-foraging *L. sericata* respond to a bi- or even multi-modal cue complex comprising at minimum both floral odour and specific floral colours (including UV). Female flies are attracted to plant

inflorescences because pollen protein and nectar (80) provide nutrients that help flies mature their oocytes. With evidence that floral protein can serve as an alternate or supplement to animal feces or carrion protein, pollen may play a major role in the foraging ecology of *L. sericata* and possibly other filth flies that, in turn, then may play a significant role as pollinators (26). This concept is supported by evidence that blow flies are highly effective at single pollen deposition for a variety of flowers (25), which is a good proxy for successful pollination.

2.6. Acknowledgements

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Table 2.1. Details on the diet of experimental *L. sericata* flies, olfactory and visual cues tested, numbers of flies tested per replicate, the duration of replicates, and numbers of replicates per experiment.

Exp. #	Test stimuli				# Flies/replicate	Sex of flies	# Replicates	Time
	Diet	Olfactory cues (**C**)	Colour cues (**C**)					
Objective 1: Investigate the effect of colour cues on attraction of L. sericata								
1	-	Honey			100	♂ & ♀	15	6 h
2	-	Honey			100	♂ & ♀	15	6 h
3	-	Honey			100	♂ &♀	15	6 h
4	-	Honey			100	♂ & ♀	15	6 h
5	-	Honey			100	♂ & ♀	15	6 h
6	-	Honey			100	♂ & ♀	15	6 h
Objective 2: Investigate the effect of Oxeye daisy floral odour on attraction of L. sericata								
7	-	C_1: 3 Daisies[1]; C_2: 3 Stems[2]			100	♂ & ♀	15	6 h
Objective 3: Investigate potential interaction between colour cues and Oxeye daisy olfactory cues on attraction of L. sericata								
8	-	C_1: 3 Daisies[1]; C_2: Empty			100	♂ & ♀	10	6 h
9	-	C_1: Empty; C_2: 3 Daisies[1]			100	♂ & ♀	10	6 h
10	-	C_1: 3 Daisies[1]; C_2: 3 Daisies[1]			100	♂ & ♀	10	6 h
11	-	C_1: Empty; C_2: Empty			100	♂ & ♀	10	6 h
Objective 4: Determine whether L. sericata females can mature their oocytes on a pollen diet								
12	Sugar	-	-		30	♀	9	13 days
13	10 Daisies/day	-	-		30	♀	9	13 days
14	Pollen[3] (0%)[4]	-	-		30	♀	9	13 days
15	Pollen[3] (20%)[4]	-	-		30	♀	9	13 days
16	Pollen[3] (30%)[4]	-	-		30	♀	9	13 days
17	Milk powder	-	-		30	♀	9	13 days

[1]Oxeye daisy inflorescence on 1-cm long stem; [2]1-cm long stem without inflorescence; [3]honey bee-collected pollen; [4]% moisture content

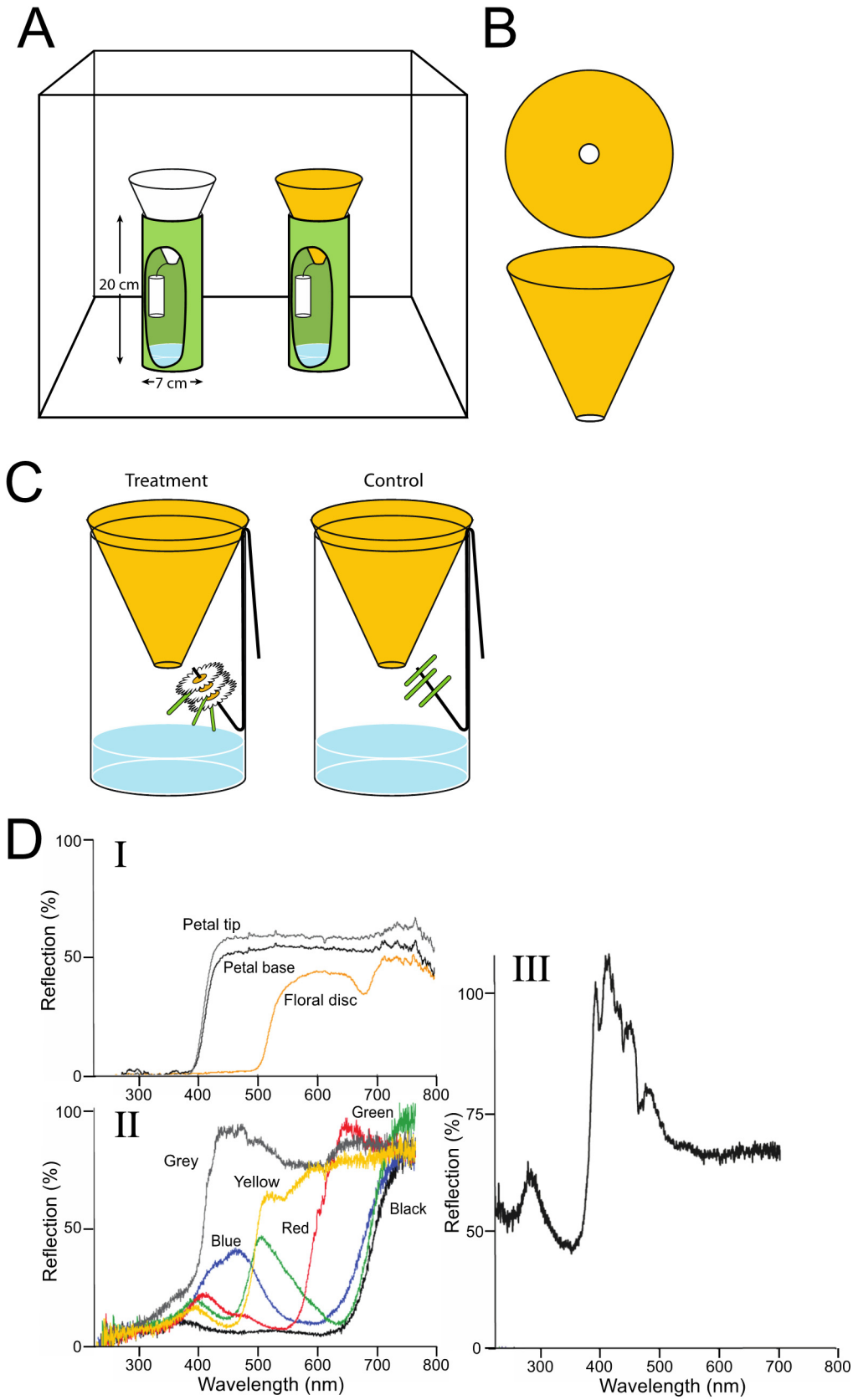
A
20 cm
7 cm
B
C
Treatment
Control
D
I
Petal tip
Petal base
Floral disc
Reflection (%)
100
50
0
300
400
500
600
700
800
II
Grey
Green
Yellow
Black
Red
Blue
Wavelength (nm)
III
75

Figure 2.1. **Graphical illustrations of experimental designs. (A, B) Design of two-choice laboratory experiments (see Table 1) with inverted bottle traps (see methods for detail), consisting of a green trap base and a funnel-like trap top covered with paper of a particular test colour, and baited with honey (A), or with three freshly-cut Oxeye daisy inflorescences on 1-cm long stems or three corresponding stems (C) as the olfactory cues; (D) representative (*n* = 5 each) spectral reflectance profiles from (I) Oxeye daisy inflorescences (floral disc, petal tip, petal base), (II) yellow, white, red, blue, green, or black construction papers tested in colour choice experiments, and (III) UV-reflective paper; the colour of each reflectance curve in I-III corresponds to the colour of the material measured; in I and III, black curves represent UV reflections.**

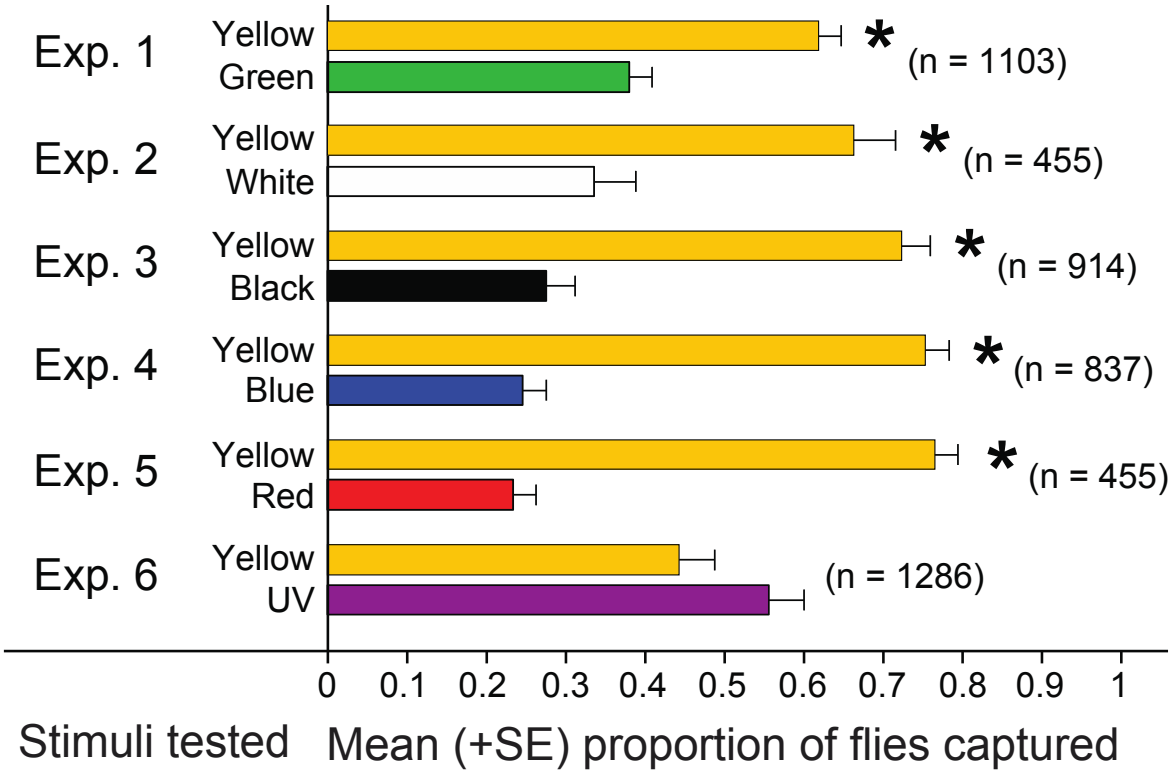

Figure 2.2. **Effect of colour cues on attraction of *Lucilia sericata.* Mean proportions of 1-, 2-, and 3-day-old males and females captured in experiments 1-6 (*n* = 15 each; Table 1) in inverted bottle traps (Fig. 1A) that were baited with a generic floral scent (honey) and with a specific colour cue (Fig. 1D) covering the inner surface of the trap funnel. In each experiment, the number in parenthesis indicates the total number of flies captured, and an asterisk (*) on a bar indicates a significant preference for the test stimulus (Wilcoxon signed rank test, $p < 0.05$).**

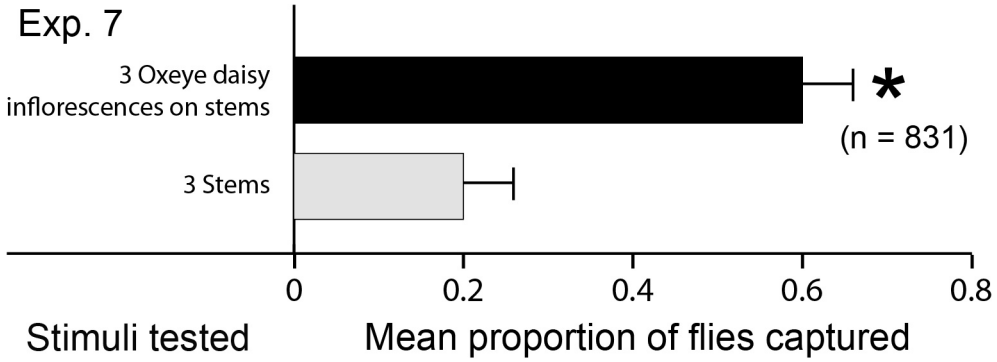

Figure 2.3. **Effect of floral odour on attraction of *Lucilia sericata.* Mean proportion of 36-h-old, females and males captured in experiment 7 (*n* = 15; Table 1) in two inverted bottle traps (Fig. 1A,B) with yellow trap funnels that were baited with either three freshly-cut Oxeye daisy inflorescences on 1-cm long stems or three corresponding stems (Fig. 1C). The number in parenthesis indicates the total number of flies captured, and the asterisk (*) indicates a significant preference for the test stimulus (Z = -2.95, df = 1, *p* = 0.0028).**

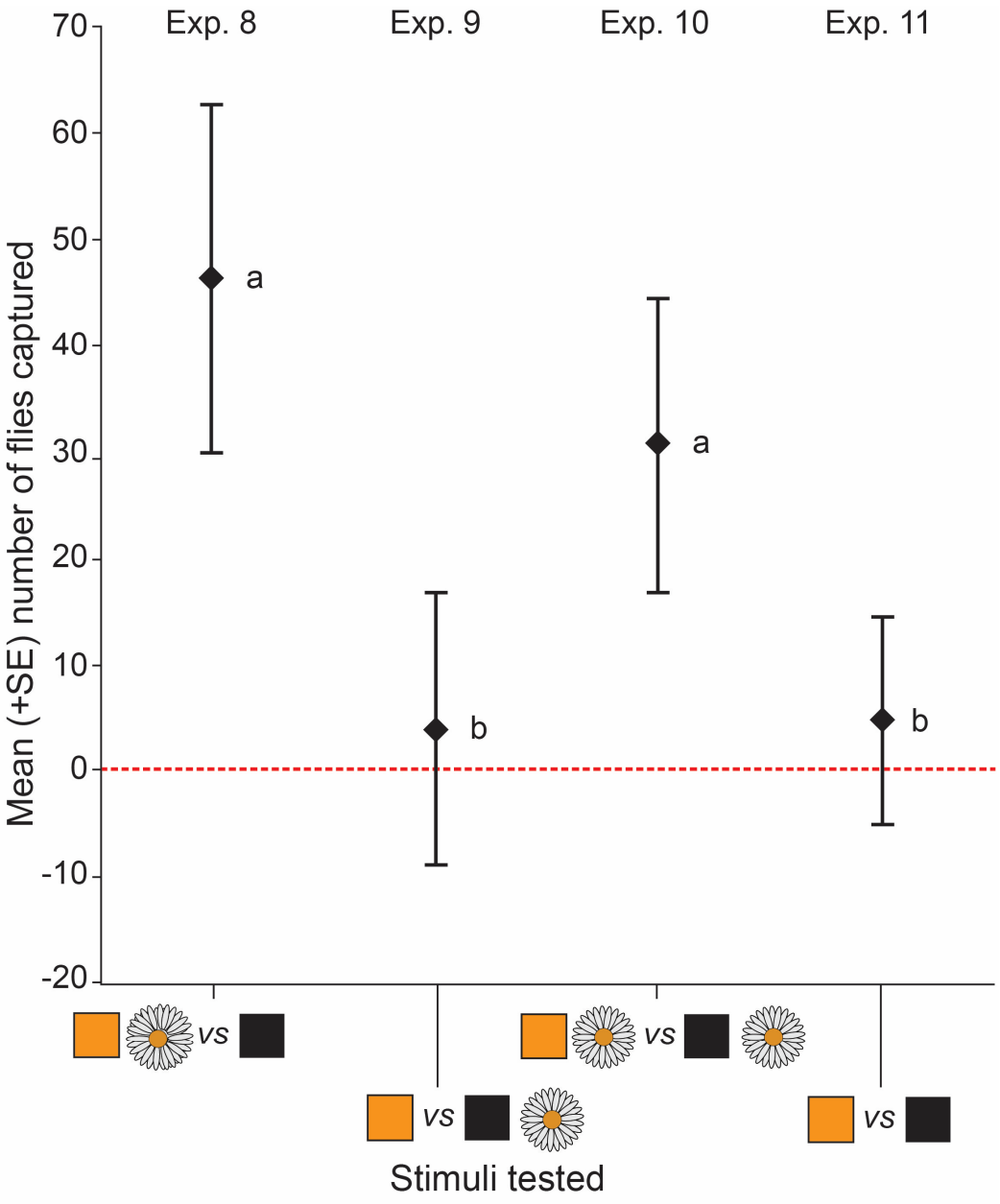
Exp. 8
Exp. 9
Exp. 10
Exp. 11
70
60
50
40
30
20
10
0
-10
-20
Mean (+SE) number of flies captured
a
b
a
b
vs
vs
vs
vs
Stimuli tested

Figure 2.4. **Interactions between visual and olfactory cues on attraction of *Lucilia sericata.* Mean number of females and males captured in experiments 8-11 (*n* = 10 each; Table 1) in paired bottle traps (Fig. 1A,B) baited with the following cue combinations: Exp. 8: Yellow with Oxeye daisy inflorescence (Fig. 1C) *versus* Black with Oxeye daisy inflorescence; Exp. 9: Yellow alone *versus* Black alone; Exp. 10): Yellow alone *versus* Black with Oxeye daisy inflorescence; and (Exp. 13): Yellow with Oxeye daisy inflorescence *versus* Black alone. Replicates of all experiments were run in parallel but those for experiment 9 (which tested the effect of colour only) were run in a separate room. Flies significantly preferred traps with yellow funnel tops (χ^2_1 (1, N = 40) = *22.83,* $p < 0.001$) and traps baited with Oxeye daisy inflorescence odour (χ^2_1 (1, N = 40) = 22.8, $p = 0.003$) but there was no interaction between colour and odour (χ^2_1 (1, N = 40) = 0.018, $p = 0.894$).**

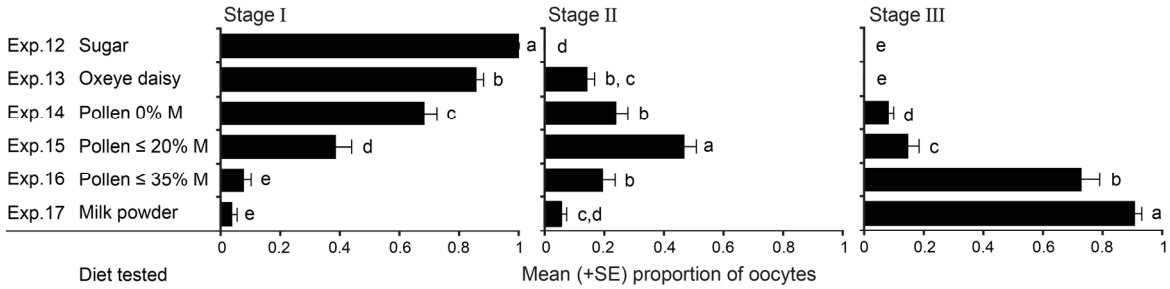

Figure 2.5. **Effect of diet on the ability of *Lucilia sericata* females to mature their oocytes. To assess the effect of diet [sugar (negative control; Exp.12), Oxeye daisy pollen from fresh inflorescences (Exp. 13), honey bee-collected pollen with 0%, ≤20% or ≤35% moisture content (Exps. 14-16), and milk powder (positive control, Exp. 17) (*n* = 9 each; Table 1) on the ability of *L. sericata* females to mature their oocytes, we scored 10 phases of ovary development according to Adams & Reinecke (47) and grouped phases into three main stages: (I) phases 0-3: oocytes with dividing cells; (II) phases 4-9: oocytes with yolk sac, and (III) phase 10: mature and chorionated eggs. Diet had a significant effect on ovarian maturation ($F_{2, 147} = 153.62$, $p < 0.0001$). Within each of stage I, II and III, bars with different letters indicate significant differences in the mean proportions of fly oocytes at that stage based on diet (Tukey's HSD: $p < 0.05$).**

Chapter 3.

Acquired Smell? Mature Females of the Common Green Bottle Fly shift Semiochemical Preferences from Feces Feeding Sites to Carrion Oviposition Sites

Brodie, B.S., T. Babcock, R. Gries, and G. Gries. A very similar (manuscript) version of this Chapter is currently accepted in the Journal of Chemical Ecology

3.1. Abstract

We investigated foraging decisions by adult females of the common green bottle fly, *Lucilia sericata,* in accordance with their physiological state. When we gave female flies a choice between visually occluded fresh canine feces (feeding site) and a CO_2-euthanized rat (carrion oviposition site), 3-d-old "protein-hungry" females responded equally well to feces and carrion, whereas protein-fed gravid females with mature oocytes responded only to carrion, indicating differential resource preferences based on the flies' physiological state. With dimethyl trisulfide (DMTS) already known to attract gravid *L. sericata* females to carrion, we analyzed headspace volatiles from canine feces by gas chromatographic-electroantennographic detection (GC-EAD) and GC-mass spectrometry. Of 17 feces odourants that elicited responses from fly antennae in GC-EAD recordings, a blend of indole and one or more of the alcohols phenol, *m-/p*-cresol and 1-octen-3-ol proved in bioassays as attractive to flies as canine feces. Unlike young females, gravid females are challenged to locate carrion oviposition resources and to distinguish between fresh and aging resources, the latter being possibly detrimental to their offspring. Gravid female *L. sericata* appear to accomplish this task, in part, by responding to trace amounts of DMTS that emanate from fresh carrion and by discriminating against carrion as soon it begins to produce appreciable amounts of indole, which is the second most abundant

semiochemical in fresh canine feces and apparently serves as an indicator of a food rather than oviposition resource. Our results emphasize the importance of studying foraging decisions by flies in accordance with their physiological stage.

3.2. Introduction

Insects undergo significant morphological and physiological changes as they progress through developmental stages. Depending on the nutritional need of each stage, they then seek and exploit specific resources (Stockhoff 1993; Hochuli 2001). For example, emergent mosquito (Diptera: Culicidae) females seek floral nectar to obtain energy (Smith and Gadawski 1994; Gary and Foster 2006), blood hosts to mature their oocytes (Foster and Takken 2004; Qiu et al. 2011; Verhulst et al. 2011), and standing water to oviposit (Osgood 1971; Olagbemiro et al. 2004; Navarro-Silva et al. 2009; Barbosa et al. 2010).

Adult blow flies (Diptera: Calliphoridae) rely on floral nectar, animal feces, and carrion for survival and reproduction (Erzinclioglu 1996). Each type of resource presents a specific nutritional or reproductive benefit to blow flies; floral nectar provides carbohydrates (Norris 1965; Grinfel'd 1955), feces are rich in protein (Hanski 1987; Linhares and Avancini 1989; Stoffolano et al. 1995), and carrion commonly serves as an oviposition site (Norris 1965; Byrd and Castner 2010). Whether young and gravid flies respond similarly to each type of resource and readily distinguish between them based on their odour profiles has hardly been investigated (but see Brodie et al. 2014).

Animal feces and manure are particularly odiferous protein sources. For example, >140 volatiles and gases emanate from pig manure (Yasuhara and Fuwa 1979; Spoelstra 1980; Yasuhara et al. 1984; O'Neill and Phillips 1992; Chen et al. 1994; Le et al. 2008), three of which [3-methylbutanoic acid, indole, dimethyl trisulfide (DMTS)] in combination are attractive to house flies, *Musca domestica* (Cossé and Baker 1996) (Diptera: Muscidae). Similarly, of the many volatiles that emanate from bird droppings, five (ammonia, methylamine, dimethylamine, trimethylamine, and 1-pyrroline) have been shown to attract tephritid fruit flies (Epsky et al. 1997; Robacker et al. 2000).

Blow flies frequently visit animal feces to obtain nutrients but oviposit only on fresh carrion (Norris 1965; Hanski 1987; Yang and Shiao 2012). Gravid - but not recently eclosed - females of the common green bottle fly, *Lucilia serricata*, orient towards fresh

carrion in response to DMTS (Brodie et al. 2014), but DMTS also emanates from feces, indicating that there must be additional semiochemicals that help blow flies distinguish at long range between these different types of resources. Some of these semiochemicals appear to evolve over time because aging carrion is rejected by gravid blow flies as a suitable oviposition site (Hall and Doisy 1993; Archer and Elgar 2003; George et al. 2012). The semiochemical(s) mediating the foraging shift by maturing flies from feces to fresh carrion, or rendering aging carrion unsuitable for oviposition, are not yet known. Identifying them and demonstrating their effect on the flies' behaviour are the major objectives of this study.

We worked with *L. sericata as* a representative blow fly species in the northern hemisphere (Hall 1948; Hall and Townsend 1977) and as an early responder to animal carrion (Davis 1928; Cragg and Hobart 1955; Cragg 1956; Hall and Doisy 1993; Byrd and Castner, 2010). We focused on the response of female flies because females undergo significant physiological changes as they mature and develop their oocytes and - unlike males - must find opposition sites to lay their eggs. We used recently deceased Norway rats, (*Rattus norvegicus* Berkenhout), and mice, (*Mus musculus* Linnaeus), as oviposition sites because they are commonly exploited by *L. sericata* and are sufficiently small to be deployed as baits in laboratory and field bioassays. We selected canine feces as a proven attractive protein source to flies (Lawson and Gemmell, 1990) because it could readily be scooped up at the very moment it was expelled by dogs.

To unravel the mechanism(s) underlying the foraging shift by maturing flies from fresh feces to fresh carrion, and the females' rejection of aged carrion, our specific objectives were to: (1) compare the attractiveness of fresh canine feces and fresh rat carrion to young and gravid flies; (2) obtain head space volatile (HSV) extract from fresh feces and bioassay its attractiveness to flies; (3) identify candidate semiochemicals in bioactive HSV extracts; (4) determine the key semiochemical(s) in HSV extracts; (5) monitor over time changes in odour profile of aging mouse carrion, and (6) test the effect of key semiochemical(s) on the acceptance or rejection of fresh mouse carrion.

3.3. Methods and Materials

3.3.1. Source of rodents and flies

Norway rats and mice were purchased by, and housed in, the Animal Care Facility of Simon Fraser University (ACF-SFU) or were purchased from a retailer (CTC Predator Feed, Duncan, BC, Canada) and CO_2-euthanized in the ACF-SFU (Permit # 1042-12). Each rat was CO_2-euthanized 5 h prior to testing and incised (Brodie et al. 2014) to simulate the death of an injured rat.

The test feces originated from two healthy mix-breed dogs (5 and 9 years old). The dogs diet consisted of kibble (Science Diet® Lamb Meal and Rice Formula and Nutro ®, respectively), and the occasional handful of table scrapes, and grass. Feces was collected fresh daily, combined, and homogenized to standardize odour cues, and was tested within 6 h of defecation.

Bioassays were conducted with flies reared in SFU's insectary under a L16:D8 photoperiod, at 30- to 40-% relative humidity and a temperature range of 23-25 °C. Every 12 months, the colony was re-started using approximately 50 wild-captured gravid female flies and increasing the colony to about 5000 flies at specific times depending on experimental requirements

3.3.2. General bioassay procedure

Two groups of flies that differed in age and reproductive status were tested in bioassays: (1) young, 3- to 5-d-old females, and (2) aged, 10- to 12-d-old (gravid) females. Considering that recently eclosed young flies seek primarily nectar carbohydrates (Brodie, unpubl. data) and may have only temporary access to ephemeral animal feces, and that gravid flies have consumed plenty of protein to mature theirs eggs (Barton Browne 2001), the group of young flies was given access only to water and sugar, whereas the group of aged gravid flies was provided with proteinaceous milk and liver powder (Sigma Aldrich, St. Louis, MO, USA) in addition to sugar and water.

For each experimental replicate, 50 cold-sedated females (Table 1) were placed into an aluminum wire mesh cage (61 × 61 × 61 cm; Fig. 1a) and given 24 h to acclimate before the bioassay. Taking into account that the response propensity of flies can vary among days and thus make experimental treatment effects less apparent, the same number of replicates of each treatment in experiments 1-4, 7-12, 13-18, and 19-22 was run concurrently (in parallel) on any given day, with treatments being applied to each cage in random order. Flies were tested only once for their response to a particular experimental treatment.

The test stimuli for laboratory experiments 1-4 are described under objective 1 (see below). For laboratory experiments 5-22, each test stimulus was placed on the bottom of a white paper Solo cup (0.5 L; 9 cm × 8.5 cm) (Solo®, Lake Forest, IL, USA), which was then covered with 2-ply white cheesecloth (VWR, Radnor, PA, USA) to standardize visual cues (Fig. 1a). Each semiochemical treatment stimulus was prepared in pentane and pipetted in 30-µl aliquots onto white filter paper (55 mm diam; Springfield Mill, UK), with equal amounts of pentane (30 µl) as the solvent control stimulus. For all tests, one randomly assigned treatment and control cup were placed 10 cm apart from each other in the center of the cage. Immediately thereafter, the number of flies that alighted on the cheesecloth of each cup was recorded for 5 min and averaged across all replicates.

Objective 1: Compare the attractiveness of fresh canine feces and fresh rat carrion to young and gravid females

Experiments 1-4 (Table 1) offered young females (Exps. 1, 2) and gravid females (Exps. 3, 4) a choice between two Ziploc® plastic containers (828 mL) (SC Johnson, Racine, WI, USA) covered in 2-ply cheese cloth to standardize visual cues, containing in one set canine feces (10 g) or nothing (empty control), and in another set rat carrion or nothing, randomly assigning test stimuli to opposite corners of the bioassay cage. Ziploc® plastic containers instead of Solo cups were used for experiments 1-4 to house the large rats (♂ mean = 463 g; ♀ mean = 224 g).

Objective 2: Obtain head space volatile (HSV) extracts from fresh canine feces and bioassay its attractiveness to flies

After placing a homogenized 10-g sample of fresh canine feces in a Pyrex® glass chamber (34 cm high × 12.5 cm wide), a pump drew charcoal-filtered air at 0.5 l min^{-1} for 5 h through the chamber and a glass column (6 mm outer diameter × 150 mm) containing 200 mg of Porapak-Q™ (50-80 mesh) adsorbent (Byrne et al. 1975). Feces-derived headspace volatiles captured on Porapak-Q were desorbed in sequence with pentane (1 mL) and ether (1 mL). Pentane and ether extracts were concentrated and combined such that 30-µL aliquots of HSV extract contained 10 gram-hour equivalents of feces volatiles (the amount of volatiles given off by 10 g of feces during 1 h)

In experiment 5 (Table 1), aliquots of this HSV extract at 10 gram-hour equivalents were then tested for their attractiveness to young flies, following the general experimental design described above.

Objective 3: Identify candidate semiochemicals in bioactive HSV extracts

Aliquots of Porapak-Q HSV extract of canine feces were analysed by gas chromatographic-electroantennographic detection (GC-EAD) and GC-mass spectrometry (MS), with procedures and equipment previously described in detail (Arn et al. 1975; Gries et al. 2002). For GC-EAD recordings (n = 10), an antenna was carefully pulled from the head of a 3- to 5-d-old female fly and suspended between two glass capillary electrodes (1.0 × 0.58 × 100 mm) (A-M Systems, Carlsborg, WA, USA) filled with saline solution (Staddon and Everton 1980). Volatile components that elicited responses from at least seven out of 10 antennae were considered candidate semiochemicals. The Hewlett Packard 5890 gas chromatograph (GC) was fitted with a DB-5 GC column [30 m × 0.32 mm inner diameter (i.d.); J&W Scientific, Folsom, CA, USA)]. Helium was used as the carrier gas (35 cm s-1) with the following temperature program: 50 °C for 5 min, 20° C min-1 to 280 °C. The injector port and flame ionization detector (FID) were set at 250 °C. Candidate semiochemicals were analyzed by a Saturn 2000 Ion Trap GC-MS operated in full-scan electron impact mode and fitted with a DB-5 GC-MS column (50 m × 0.25 mm i.d.). Helium was used as the carrier gas (35 cm s-1) with the following temperature program: 50 °C for 1 min, 10 °C min-1 until 280 °C (10 min). The injector port and ion trap were set at 250 °C and 260 °C, respectively. Components that elicited responses from fly

antennae were identified by comparing their retention indices (Van den Dool and Kratz 1963) and mass spectra with those reported in the literature (Jennings and Shibamoto 1980; Adams 1989) and with those of authentic standards.

To confirm the structural assignment of candidate semiochemicals and to prepare synthetic blends (see objective 4), the following compounds were purchased: propanoic acid (>99.5% chemically pure), 2-methylpropanoic acid (99%), butanoic acid (>99%), 2-methylbutanoic acid (>98%), 3-methylbutanoic acid (99%), phenylacetaldehyde (90%), nonanal (95%), decanal (>95%), phenol (>95%), 1-octen-3-ol (>98%), meta- and para-cresol (99%), sulcatone (>98%), geranylacetone (>97%), indole (>99%), DMTS (>95%) (all Sigma Aldrich, St. Louis, MO, USA), and (*E*)-2-octenal (Bedoukian, Danbury, CT, USA).

Objective 4: Determine the key semiochemical(s) in bioactive HSV extracts

Experiment 6 (Table 1) tested a synthetic blend (SB) of all candidate semiochemicals in HSV extracts of feces that had elicited responses from fly antennae in GC-EAD recordings (see Results). The SB included propanoic acid, 2-methylpropanoic acid, butanoic acid, 2-methylbutanoic acid, 3-methybutanoic acid, phenylacetaldehyde, (*E*)-2-octenal, nonanal, decanal, phenol, 1-octen-3-ol, *meta*- and *para*-cresol, sulcatone, geranylacetone, indole, and dimethyl trisulfide (DMTS). As *m*- and *p*-cresol could not be separated they were both included in the SB. All components were prepared in pentane at ratios equivalent to those found in GC-MS analyses of HSV extracts and were tested at 10 gram-hour equivalents, following the general bioassay procedure described above.

With evidence that HSV extract of fresh canine feces and the SB were both effective in attracting flies (see 'Results'), the next set of experiments was designed to determine the essential semiochemical(s) mediating the response of flies to the SB. Accordingly, parallel-run experiments 7-12 (Table 1) tested the SB (experiment 7), and SBs that lacked groups of organic chemicals such as alcohols (experiments 8), aldehydes (experiment 9), ketones (experiment 10), acids (experiment 11), and N- or S-containing compounds (experiment 12). All SBs were tested following the general bioassay procedure described above.

With evidence that an alcohol and a N- or S-containing compound (DMTS or indole) are key feces semiochemicals (see Results), experiments 13-18 (Table 1) then tested the SB (experiment 13) and SBs lacking a specific alcohol such as phenol (experiments 14), 1-octen-3-ol (experiments 15) or *m/p*-cresol (experiments 16), or lacking DMTS (experiment 17) or indole (experiments 18). All SBs were tested following the general bioassay procedure described above.

With evidence that indole but neither DMTS nor one specific alcohol are key feces semiochemicals (see Results), experiments 19-22 (Table 1) investigated potential interactive effects between components, testing the SB (experiment 19), the three alcohols combined (phenol, 1-octen-3-ol, *m-/p*-cresol (experiments 20), indole alone (experiment 21), and a mixture of the alcohols and indole (experiment 22). All synthetic stimuli were tested following the general bioassay procedure described above.

Objective 5: Monitor over time changes in odour profile of aging mouse carrion

To quantify the amount of indole and DMTS in headspace volatiles of mouse carrion, 10 CO_2-euthanized mixed sex mice in each of 3 replicates were placed in a Pyrex® glass aeration chamber, capturing their headspace volatiles on Porapak-Q (see Objective 2 and Table 1 for detailed methods). Following the first 16-h interval of volatile capture, the Porapak-Q volatile traps were replaced every 12 h up to 76 h, and aliquots of Porapak-Q extracts were analyzed by GC-MS (see Objective 3 and Table 1 for detailed methods), using hexyl acetate as an internal standard.

Objective 6: Test the effect of key semiochemical(s) on the acceptance or rejection of mouse carrion

With evidence that young and aged (gravid) flies differ in their resource preferences (see Results), and that indole is absent from fresh carrion but essential for attraction of young flies to canine feces (see Results), experiments 23-25 were designed to test the effect of fresh canine feces, or of indole specifically, on the response of flies to fresh mouse carrion. Laboratory experiment 23 and field experiment 24 (n = 10; Table 1) tested the response of aged (13-d-old) flies and wild type flies, respectively, to one mouse carrion at the bloat stage (24 h post CO_2-euthanazition at room temperature) and the

same type of mouse carrion in combination with fresh canine feces (20 g) applied to the outside of a mesh bag (50 × 63 mm; Fig. 1c) enclosing the carrion. The mesh bag was suspended from a wire above a soap water mote inside an Oak Stump trap covered with brown paper to occlude test stimuli. For each laboratory experimental replicate, 50 cold-sedated female flies were allowed to acclimate for 1 h before the two traps were placed 0.45 cm apart from one another in opposite corners of the bioassay cage. After a 1-h experimental period, the traps were removed, captured flies counted, and their reproductive status (non-gravid, gravid) determined by ovary dissection (Adams and Reinecke 1979).

Field experiment 24 (13-15 September 2014; Table 1) was conducted on a berry farm (49°01'31.55"N, 122°18'43.61"W) next to a poultry farm in Abbotsford, BC, Canada. Testing the same two stimuli as in experiment 23 (mouse carrion with or without canine feces), Oak Stump traps were suspended 0.5 m above ground from a fence in randomized complete blocks with 10-m inter-trap spacing within and between blocks. Near the end of the photophase in each of two consecutive days, the number, sex, and physiological status (see above, exp. 23) of captured specimens of each fly species were determined. Combined data for days 1-2 are reported.

Field experiment 25 (21-23 September 2014; Table 1) had the same design as experiment 24, but the freshly deceased mouse carrion in each trap was combined with a cotton ball (Fig. 1c) which was attached to the mesh bag enclosing the carrion and was impregnated with ether (control) or indole (100 µg) dissolved in ether.

3.3.3. Statistical analyses

All data were analyzed with JMP 11® (SAS Institute Inc.). In experiments 1-4, 7-12, 13-18, and 19-22, the mean proportions of fly landings on paired Solo cups were analyzed by one-tailed t-test, expecting a preferred stimulus to induce >65% of the fly responses. Data (mean cumulative number of fly landings on treatment stimuli) in experiments 7-12 and 19-22 were log-transformed and evaluated for normality using a Q-Q plot. Within experiments 7-12, 13-18, and 19-22, respectively, the flies' alighting responses on treatment stimuli were analyzed by Analysis of Variance followed by

Tukey's HSD test. The flies' alighting responses on treatment stimuli in experiments 5 and 6 were compared by a Wilcoxon test. Data in each of experiments 23-25 were analysed with a non-parametric Wilcoxon signed rank test.

3.3.4. Results

Objective 1: Compare the attractiveness of fresh canine feces and fresh rat carrion to young and gravid females

Fresh canine feces received >65% of the alighting responses from young females (Fig. 2; Exp. 1: *df*=9, t=30.84, *P*<0.001) but not from gravid females (Fig. 2; Exp. 3: *df*=9, t=-1.394, *P*=0.896), indicating that fresh canine feces is attractive only to young flies. In contrast, fresh rat carrion received >65% of the alighting responses from both young females (Fig. 2; Exp. 2: *df*=9, t=33.99, *P*<0.001) and gravid females (Fig. 2; Exp. 4: *df*=9, t=47.12, *P*<0.001), indicating that fresh rat carrion is attractive to both young and gravid flies.

Objective 2: Obtain head space volatile (HSV) extract from fresh feces and bioassay its attractiveness to flies

Aliquots of Porapak Q HSV extract of fresh canine feces received 62.6 ± 5.1 fly landings (>65% of the flies' landing responses; Fig. 3; Exp. 5: t=7.83, *df*=13, *P*<0.001), whereas the solvent control received only 19 ± 1.7 fly landings, indicating that essential semiochemical(s) associated with fresh canine feces were present in HSV extract.

Objective 3: Identify candidate semiochemicals in bioactive HSV extracts

In GC-EAD analyses of HSV extract of fresh canine feces, 12 odourants consistently elicited responses from fly antennae (Fig. 4). They were phenylacetaldehyde, (*E*)-2-octenal, nonanal, decanal, phenol, 1-octen-3-ol, *m*- and *p*-cresol, sulcatone, geranylacetone, indole, and DMTS. Antennal responses to propanoic acid, 2-methylpropanoic acid, butanoic acid, 2-methylbutanoic acid, and 3-methybutanoic acid varied greatly between runs, likely due to poor chromatography of these acids.

Objective 4: Determine the key semiochemical(s) in bioactive HSV extracts

The synthetic blend (SB) of candidate semiochemicals from fresh canine feces received 70 ± 7.25 fly landings (>65% of the flies' landing responses; Fig. 3; Exp. 6: t=6.69, *df*=9, *P*<0.001), whereas the solvent control received only 19.7 ± 1.23 fly landings, indicating that the SB contained key semiochemicals associated with canine feces. Moreover, the flies' mean response to the SB (Exp. 6: 70.0 ± 7.3 landings) and to HSV extract of feces (Exp. 5: 62.6 ± 5.1 landings) did not differ significantly (Z=140.5, *df*=1, *P*=0.380), indicating further that the SB contained all the key semiochemical(s) of canine feces.

The response of young females to the complete SB or to SBs lacking specific components differed significantly (Exps. 7-12; $F_{(5,54)}$=4.45, *P*=0.002; Fig. 5). In comparison to solvent control stimuli, the complete SB and SBs lacking acids, aldehydes or ketones all received >65% of the flies' landings responses (*df*=9 each; Exp. 7: t=7.45, *P*<0.001; Exp. 8: t=6.46, *P*<0.001; Exp. 9: t=7.09, *P*<0.001; Exp. 11: t=5.79, *P*<0.001; Fig. 5). The same type of preferential response was not evident for SBs lacking alcohols (Exp. 10: t=1.29; *P*<0.115) or lacking indole and DMTS (Exp. 12: t=-1.46; *P*<0.911), indicating that at least one alcohol as well as indole and/or DMTS mediate attraction of young females to fresh canine feces.

The response of young females to the complete SB or to SBs lacking a specific component differed significantly (Exps. 13-18; $F_{(5,54)}$=10.5, *P*<0.001; Fig. 5). In comparison to solvent control stimuli, the complete SB and SBs lacking phenol, 1-octen-3-ol, *p*/*m*-cresol, or DMTS all received >65% of the flies landing responses (*df*=9 each; Exp. 13: t=4.73, *P*=0.0011; Exp. 14: t=5.96, *P*<0.001; Exp. 15: t=2.73, *P*=0.0231; Exp. 16: t=3.82, *P*=0.0041; Exp. 17: t=7.30, *P*<0.001; Fig. 5), indicating that neither of these odourants alone mediates attraction of young females to fresh canine feces. In contrast, the SB lacking indole failed to receive >65% of the flies landing responses (Exp. 18: t=0.70, *P*=0.22; Fig. 5), indicating that the presence of indole is critically important for attracting young females to fresh canine feces.

The response of young females to test stimuli consisting of the complete SB, three alcohols (phenol, 1-octen-3-ol, *m*- and *p*-cresol), indole, or a mixture of the alcohols and

indole differed significantly (Exps. 19-22; $F_{(3,36)}$=18.03, *P*<0.001; Fig. 5). In comparison to solvent control stimuli, the complete SB, and the mixture of alcohols and indole, both received >65% of the flies landing responses (*df*=9 each; Exp. 19: t=11.81, *P*<0.001; Exp. 22: t=9.62, *P*<0.001; Fig. 5). The same type of preferential response was not evident when we tested the alcohols (Exp. 20: *df*=9, t=0.44, *P*=0.439) or indole (Exp. 21: *df*=9, t=0.04, *P*=0.489) which received 12.9 ± 3.3 fly landings and 12.6 ± 4.6 fly landings, respectively, five times fewer than the mixture of alcohols and indole, indicating synergistic attractiveness between indole and one or more alcohols.

Objective 5: Monitor over time changes in odour profiles of aging mouse carrion

During 16 hours *post mortem*, indole was detectable in only one of three replicates (10 mice carcasses per replicate) and in only small amounts (0.01 µg; Fig. 6), whereas DMTS was detectable in all three replicates, averaging 1.33 µg (Fig. 6). During subsequent 12-h sampling intervals, the amounts of both DMTS and indole released from mice carcasses increased substantially, particularly in the 42- to 52-h, 52- to 64-h, and 64-76-h *post mortem* intervals. At each sampling interval, the amount of DMTS exceeded the amount of indole by 13- to 31-times (Fig. 6).

Objective 6: Test the effect of key semiochemical(s) on the acceptance or rejection of mouse carrion

In laboratory experiment 23 with (>90%) gravid females, traps baited with both mouse carrion and canine feces captured significantly fewer females (Z=-3.745, *df*=1, *P*<0.001; Fig. 7), and significantly fewer gravid females (Z=-3.75, *df*=1, *P*<0.001; Fig. 7), than traps baited with mouse carrion alone, revealing an less attractive effect of canine feces on the response of oviposition site-seeking flies. In contrast, non-gravid females responded equally to both baits (Z=-1.793, *df*=1, *P*=0.073; Fig. 7), substantiating prior results (Fig. 2, Exps. 1, 2) that canine feces and recently deceased rat carrion are equally attractive to protein-hungry flies with immature oocytes.

The type of trap bait had a significant effect on captures of wild female flies (Z = -2.293, *df*=1; P = 0.022, Fig. 7, exp. 24). Traps baited with both mouse carrion and canine feces attracted significantly more non-gravid flies (Z = -2.973, *df* = 1, P = 0.003) than

traps baited with mouse carrion alone, indicating a preference of these flies to the proteincaceous feces resource. However, there was no significant preference for either bait by gravid flies (Z = -0.873, *df* = 1, P = 0.383).

In field experiment 25, the type of trap bait had no significant effect on the overall number of wild flies captured (Z=0.325, *df*=1, *P*=0.985, Fig. 7). However, traps baited with both mouse carrion and indole (an indicator semiochemical of feces) captured significantly fewer gravid female flies than traps baited with mouse carrion alone (Z=-2.89, *df*=1, *P*=0.0038). In contrast, captures of non-gravid females in the same-paired traps were not affected by the presence of indole (Z=-0.38, *df*=1, *P*=0.704).

3.4. Discussion

Our data demonstrate the following: (1) the physiological state of *L. sericata* females affects their resource preference: fresh canine feces attracts young (protein-hungry) flies but not aged (gravid) flies, whereas fresh rat carrion attracts both young and gravid flies; (2) attraction of young flies to canine feces is mediated by feces semiochemicals, of which indole and one or more of the alcohols phenol, *m-/p*-cresol and 1-octen-3-ol play key roles; (3) at an advanced but not at an early stage of decay mouse carrion produces indole, (4) the smell of fresh canine feces, or of indole, is off-putting to gravid females seeking oviposition sites, indicating that indole signifies the presence of animal feces (protein resources) rather than oviposition sites, or, that the carrion is at an advanced stage of decay and thus unsuitable for oviposition.

Indole was the second most abundant (18%) fecal odourant that elicited responses from *L. sericata* antennae. It has a strong fecal odour (Jensen et al. 1995) and is produced during the degradation of tryptophan, a major building block of proteins (Whitley and Thornton 2012), by intestinal bacteria such as *Escherichia coli* (Dawes 1948; Schulz and Dickschat 2007), *Enterobacter* spp., *Klebsiella* spp. (Schulz and Dickschat 2007), *Lactobaccillus* spp., and *Clostridium* spp. (Jensen et al. 1995) that are present in most animal feces. Both the relative abundance and intense odour of indole could make indole a reliable foraging cue for *L. sericata* females seeking fecal resources for protein meals.

While indole is an essential fecal semiochemical for foraging *L. sericata* females, its attractiveness hinges on the presence of one or more of the alcohols phenol, *m-/p*-cresol and 1-octen-3-ol (Fig. 5, Exps. 21, 22). Unlike indole, which has moderate volatility and thus likely serves as a long-range attractant, the rather volatile alcohols could function as close-range semiochemical attractants that help blow flies pinpoint the micro-location of a fecal resource. An analogous system was reported for the aggregation pheromone of the bark beetle *Ips typographus*, where the "heavier" pheromone component (4*S*)-*cis*-verbenol attracts beetles from long-range, whereas the more volatile pheromone component 2-methyl-3-buten-2-ol attracts beetles at short range (Bakke et al. 1977; Schlyter et al. 1987).

The feces-derived alcohols exhibit some degree of semiochemical redundancy, because omitting any one alcohol from the synthetic blend of antennally-active fecal odourants did not reduce blend attractiveness (Fig. 5, Exps. 14-16). Intense fecal odour is typically associated with recently deposited and thus moist feces, but not as much with dried-up feces, suggesting that fecal odour intensity is correlated with the moisture content of animal feces. As the volatile alcohols will dissipate most quickly, their diminishing contribution to the odour bouquet could reflect the diminishing moisture content of the feces. If so, this information would be critical to foraging blow flies that cannot feed very well on dry feces (Hanski 1987).

There are contrasting reports in the literature on the response of calliphorid flies to indole or fecal resources. Indole was not yet known to attract calliphorid flies to animal feces, but was shown to be part of a semiochemical blend that induced attraction to suboptimal oviposition sites such as larval waste from artificial rearing material (Chaudhury et al. 2014) and fleece from an oviposition host (Eisemann 1995). Conversely, indole had no effect on the attraction of blow flies in studies that investigated the attractiveness of oviposition sites rather than food sources, and that exclusively bioassayed the response of protein-fed flies (Easton and Feir 1991; Frederickx et al. 2011). The results of these previous studies and our own data indicate that age, physiological need, and reproductive status of blow flies affect their propensity to respond to semiochemicals from specific resources.

Our conclusion that fecal semiochemicals in general, and indole in particular, are off-putting to gravid, oviposition site-seeking *L. sericata* females, is supported by both laboratory and field data. Gravid *L. sericata* females were not more attracted to canine feces than they were to benign control stimuli (Fig. 2, Exp. 3), strongly preferred mouse carrion over canine feces (Fig. 7, Exp. 23), and discriminated against mouse carrion when presented in combination with indole (Fig. 7, Exp. 25). Young flies, in contrast, were strongly attracted to canine feces (Fig. 2, Exp. 1), headspace volatile extract of canine feces (Fig. 3, Exp. 5), and to various blends of synthetic fecal semiochemicals (Fig. 3, Exp. 6; Fig. 5, Exps. 7-22). Avoiding aging carrion with incipient dissemination of indole (Fig. 6) as oviposition sites may help gravid *L. sericata* females minimize adverse fitness effects. If females were to oviposit on animal carrion at later stages of decay, they would subject their offspring to predation by scavenging vertebrates or to resource competition by detritus-consuming insects, fungi and microbes (DeVault et al. 2003, 2004; Mohr and Tomberlin 2014). These phenomena might explain why gravid female flies typically do not oviposit on carrion at advanced stages of decomposition (Huntington et al. 2008). That gravid females, and other flies, still respond to these types of resources could be motivated by search for food (which liquefied carrion provides) or for mates (which often gather on or near food resources) (Archar and Elgar 2004).

In conclusion, the resource preference of *L. sericata* females depends on their physiological state: young protein-seeking *L. sericata* females are strongly attracted to feces and to carrion. Females at this physiological stage respond as readily to fresh canine feces they do to a feces semiochemical blend of indole and one or more of the alcohols phenol, *m-/p*-cresol and 1-octen-3-ol. Gravid females, in contrast, that already had many protein meals to mature their eggs are challenged to locate carrion oviposition resources and to distinguish between fresh and aging resources. Selecting recently deceased carcasses (oviposition resources) would help females minimize resource competition for their offspring. Female *L. sericata* appear to accomplish this task, in part, by responding to trace amounts of DMTS that emanate from fresh carrion and by discriminating against carrion as soon it begins to produce appreciable amounts of indole, which is the second most abundant semiochemical in fresh canine feces and apparently serves as an indicator of a food rather than an oviposition resource. Our results

emphasize the importance of studying foraging decisions by flies, and possibly by other insects, in accordance with their physiological stage.

3.5. Acknowledgements

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Table 3.1. Details on the olfactory stimuli tested, and number and duration of experimental replicates for laboratory and field responses of *Lucilia sericata* flies

Exp. No	Location	Test stimuli (**T**)	Type of flies & No of replicates				Time
			Protein-hungry (3- to 5-d-old)		Protein-fed (10- to 12-d-old)		
			♀	♂	♀	♂	
Objective 1: Compare the attractiveness of fresh canine feces and fresh rat carrion to young and gravid females							
1-4	Laboratory	**T_1:** Canine feces (10g; fresh); **T_2:** Rat carrion (fresh)	10	10	10	10	5 min
Objective 2: Obtain head space volatile (HSV) extracts from fresh canine feces and bioassay its attractiveness to flies							
5	Laboratory	**T_1:** HSV[a] extract of canine feces; **T_2:** Solvent control	14	–	–	–	5 min
Objective 3: Identify candidate semiochemicals in bioactive HSV extracts							
Objective 4: Determine the key semiochemical(s) in bioactive HSV extracts							
6, 7, 13, 19	Laboratory	**T_1:** SB[a,b]; **T_2:** Solvent control	10	–	–	–	5 min
8	Laboratory	**T_1:** SB *minus* acids; **T_2:** Solvent control	10	–	–	–	5 min
9	Laboratory	**T_1:** SB *minus* aldehydes; **T_2:** Solvent control	10	–	–	–	5 min
10	Laboratory	**T_1:** SB *minus* alcohols; **T_2:** Solvent control	10	–	–	–	5 min
11	Laboratory	**T_1:** SB *minus* ketones; **T_2:** Solvent control	10	–	–	–	5 min
12	Laboratory	**T_1:** SB *minus* [indole + DMTS][b]; **T_2:** Solvent control	10	–	–	–	5 min
14	Laboratory	**T_1:** SB *minus* phenol; **T_2:** Solvent control	10	–	–	–	5 min
15	Laboratory	**T_1:** SB *minus* 1-octen-3-ol; **T_2:** Solvent control	10	–	–	–	5 min
16	Laboratory	**T_1:** SB *minus m-/p*-cresol; **T_2:** Solvent control	10	–	–	–	5 min
17	Laboratory	**T_1:** SB *minus* DMTS; **T_2:** Solvent control	10	–	–	–	5 min
18	Laboratory	**T_1:** SB *minus* indole; **T_2:** Solvent control	10	–	–	–	5 min
20	Laboratory	**T_1:** Alcohols; **T_2:** Solvent control	10	–	–	–	5 min
21	Laboratory	**T_1:** Indole; **T_2:** Solvent control	10	–	–	–	5 min
22	Laboratory	**T_1**: Alcohols plus indole; **T_2**: Solvent control	10	–	–	–	5 min
Objective 5: monitor over time changes in odour profile of aging mice carrion							
Objective 6: Test the effect of key semiochemical(s) on the acceptance or rejection of fresh mouse carrion							
23	Laboratory	**T_1:** Mouse carrion (24-h old); **T_1** + **T_2**: Canine feces (fresh; 20g)			10		1 h
24	Field[c]	**T_1:** Mouse carrion (fresh); **T_2**: **T_1** + canine feces (fresh; 20g)					3 days
25	Field[c]	**T_1:** Mouse carrion (fresh) + ether; **T_2**: **T_1** + indole (100µg in ether)					3 days

[a]Headspace volatile (HSV) extract and the synthetic blend (SB) were tested at 10 fecal gram-hour-equivalents (volatiles released from 10 g of fresh canine feces during 1 h); [b]SB: acids [propanoic (30 ng), 2-methylpropanoic (30 ng), butanoic (300 ng), 2-methylbutanoic (300 ng), 3-methybutanoic (300 ng)]; aldehydes [phenylacetaldehyde (3 ng), (*E*)-2-octenal (3 ng), nonanal (9 ng) , decanal (9 ng)]; alcohols [phenol (1260 ng), 1-octen-3-ol (15 ng), *m*-/*p*-cresol (12 ng each)]; ketones [sulcatone (9 ng), geranylacetone (6 ng)]; indole (660 ng), dimethyl trisulfide (DMTS, 15 ng); [c]10 & 11 replicates.

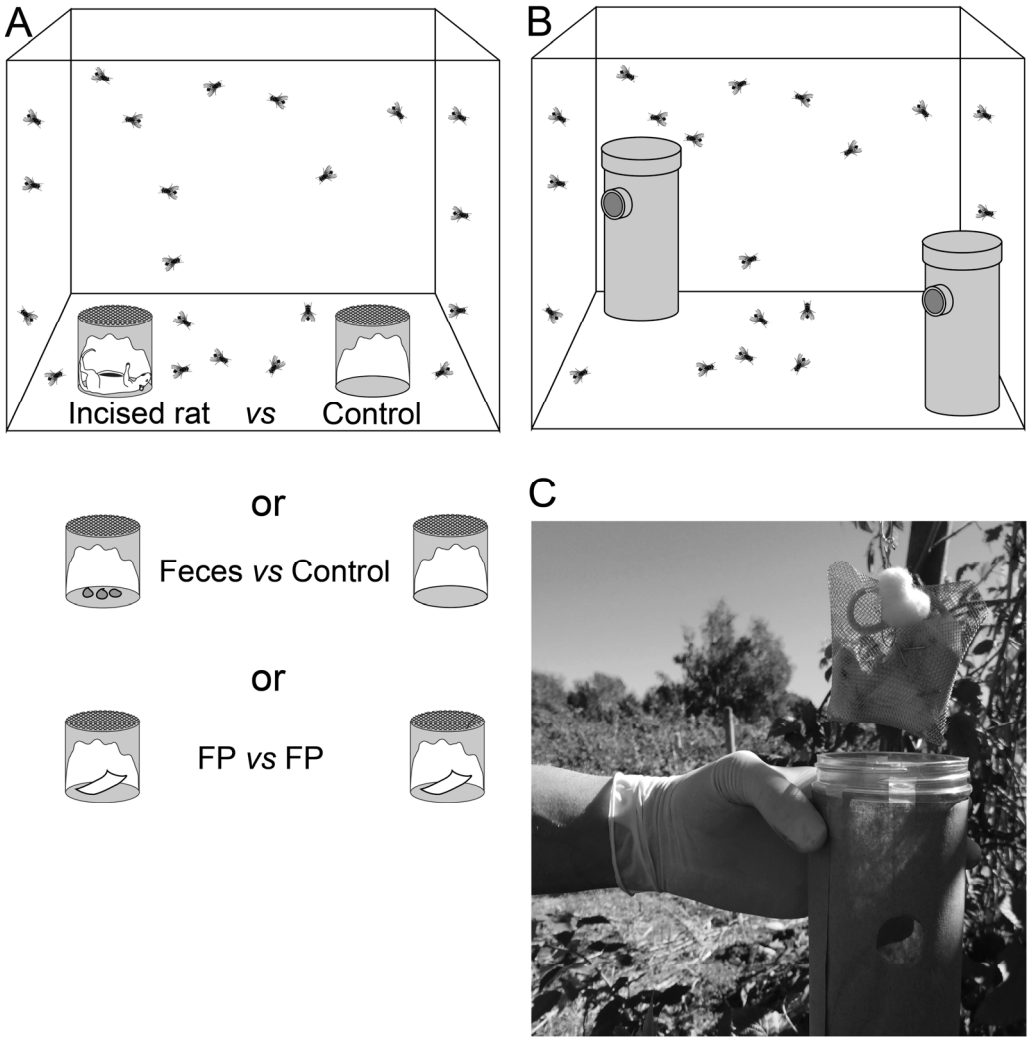

Figure 3.1. **(a) Design of laboratory experiments which recorded alighting responses of flies on paired Ziploc storage containers (experiments 1-4) and on paired Solo cups (experiments 5-22) containing (*i*) canine feces (10 g) or nothing (empty control), (*ii*) rat carrion or nothing, and (*iii*) filter paper (FP) impregnated with (synthetic) semiochemicals or a solvent control; (b) design of laboratory experiment 23 which recorded captures of flies in paired Oak Stump traps covered in brown construction paper and baited with a mouse carcass (see c) or canine feces (20g); (c) Oak Stump trap baited for field experiments 24 and 25 with a mouse carcass in a mesh bag; the white cotton ball was impregnated with indole (100 µg) dissolved in ether or an ether control.**

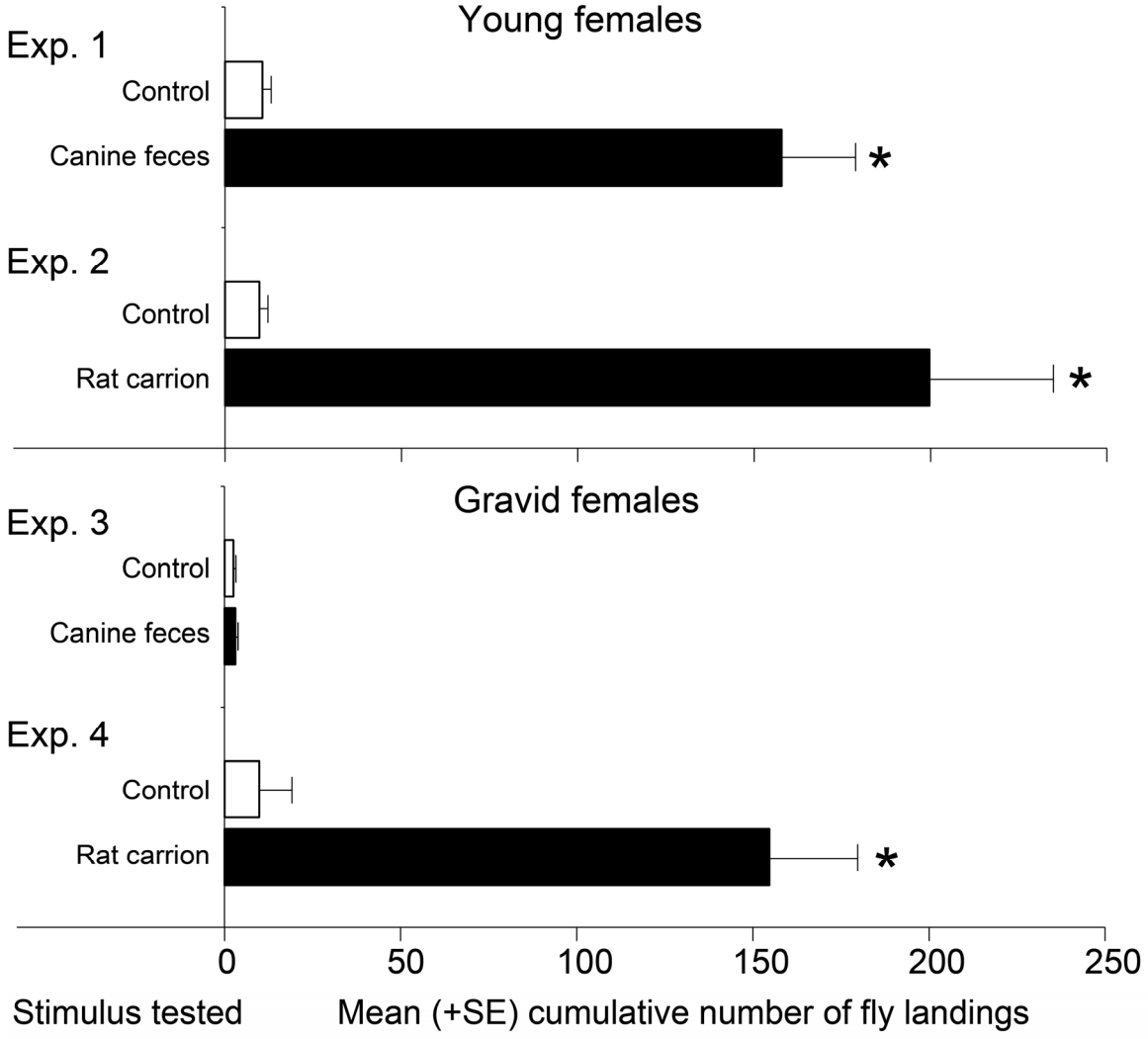

Figure 3.2. **Mean (+ SE) cumulative number of alighting responses by young and gravid females of *Lucilia sericata* in experiments1-4 (n = 10 each) on paired, cheesecloth-covered Ziploc plastic containers that were left empty (control) or baited with fresh canine feces or fresh rat carrion (Fig. 1a). In each of experiments 1, 2 and 4, the asterisk (*) denotes that the treatment stimulus received significantly more than 65% of the flies' alighting responses (one-tailed t-test; Exp. 1: t=30.84, *df*=9, *P*<0.001; Exp. 2: t=33.99, *df*=9, *P*<0.001; Exp. 4: t=47.12, *P*<0.001).**

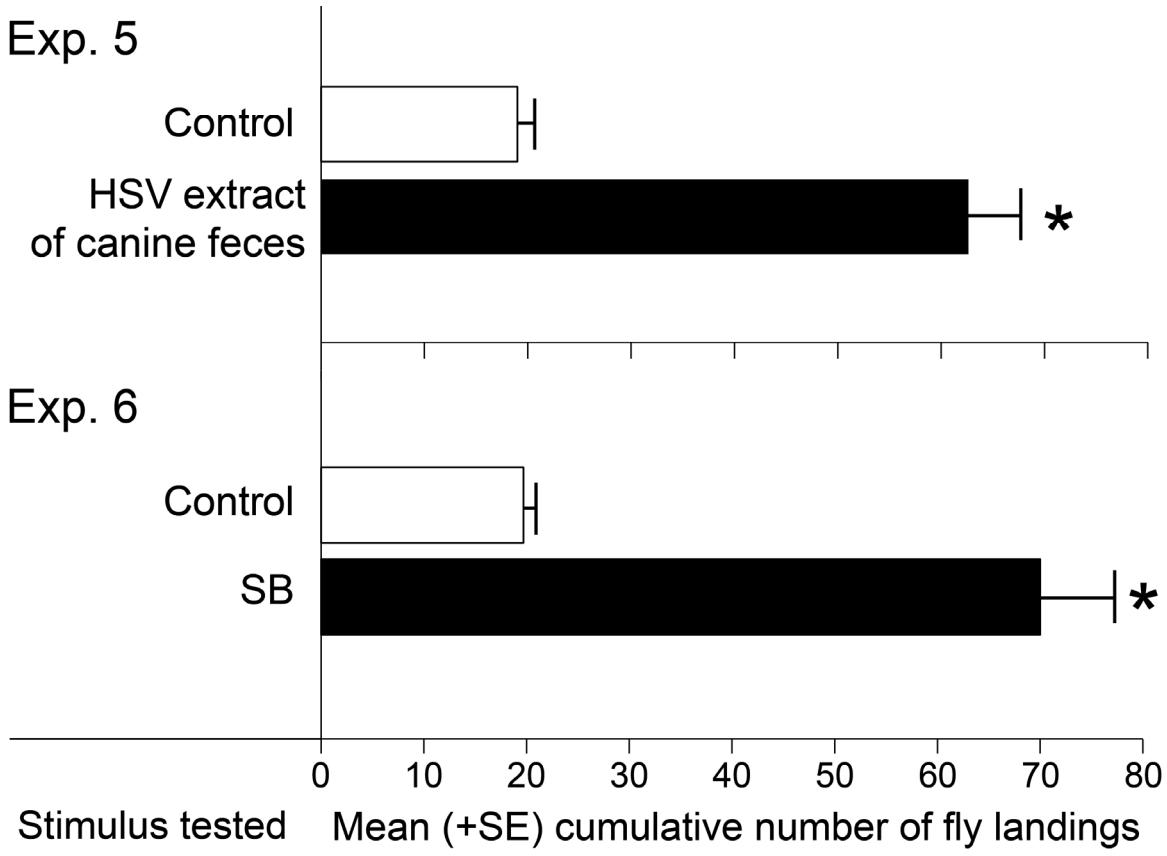

Figure 3.3. **Mean (+ SE) cumulative number of alighting responses by young females of *Lucilia sericata* in experiment 5 (n = 14) and 6 (n = 10) on paired, cheesecloth-covered Solo cups (see Fig. 1a) containing filter paper impregnated with (*i*) headspace volatile (HSV) extract of fresh canine feces in pentane/ether at 10-gram-hour equivalents (amount of volatiles given off 10 g of feces during 1 h) (Exp. 5), and (*ii*) a synthetic blend (SB) of candidate semiochemicals (see Fig. 3, Table 1) in pentane/ether or pentane/ether (Exp. 6). In experiments 5 and 6, the asterisk (*) denotes that the treatment stimulus received significantly more than 65% of the flies' alighting responses (one-tailed t-test; Exp. 5: t=7.83, *df*=13, *P*<0.0001; Exp. 6: t=6.69, *df*=9, *P*<0.0001).

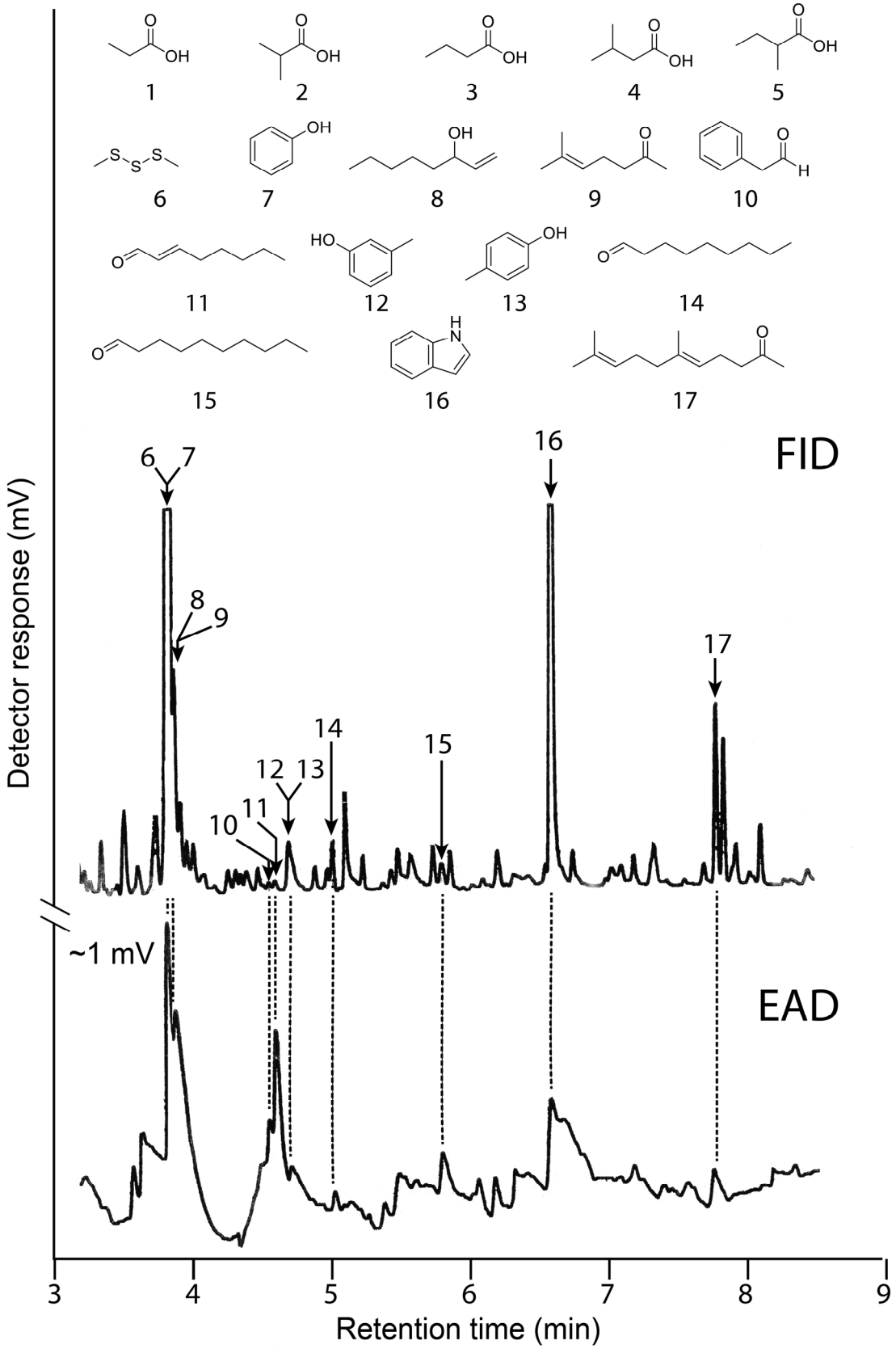
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
FID
EAD
~1 mV
Detector response (mV)
Retention time (min)
3
4
5
6
7
8
9

Figure 3.4. **Representative recording of the responses of a gas chromatographic flame ionization detector (FID) and an electroantennographic detector (EAD: female *Lucilia sericata* antenna) to an aliquot of Porapak Q headspace volatile extract of fresh canine feces. Antennal responses to propanoic acid (a), 2-methylpropanoic acid (b), butanoic acid (c), 3-methybutanoic acid (d), and 2-methylbutanoic acid (e) varied greatly between runs due to poor chromatography of these acids and are not depicted here. EAD-active odourants f-q were: dimethyl trisulfide (f), phenol (g), 1-octen-3-ol (h), sulcatone (i), phenylacetaldehyde (j), (*E*)-2-octenal (k), *meta*- and/or *para*-cresol (l, m), nonanal (n), decanal (o), indole (p), and geranylacetone (q).**

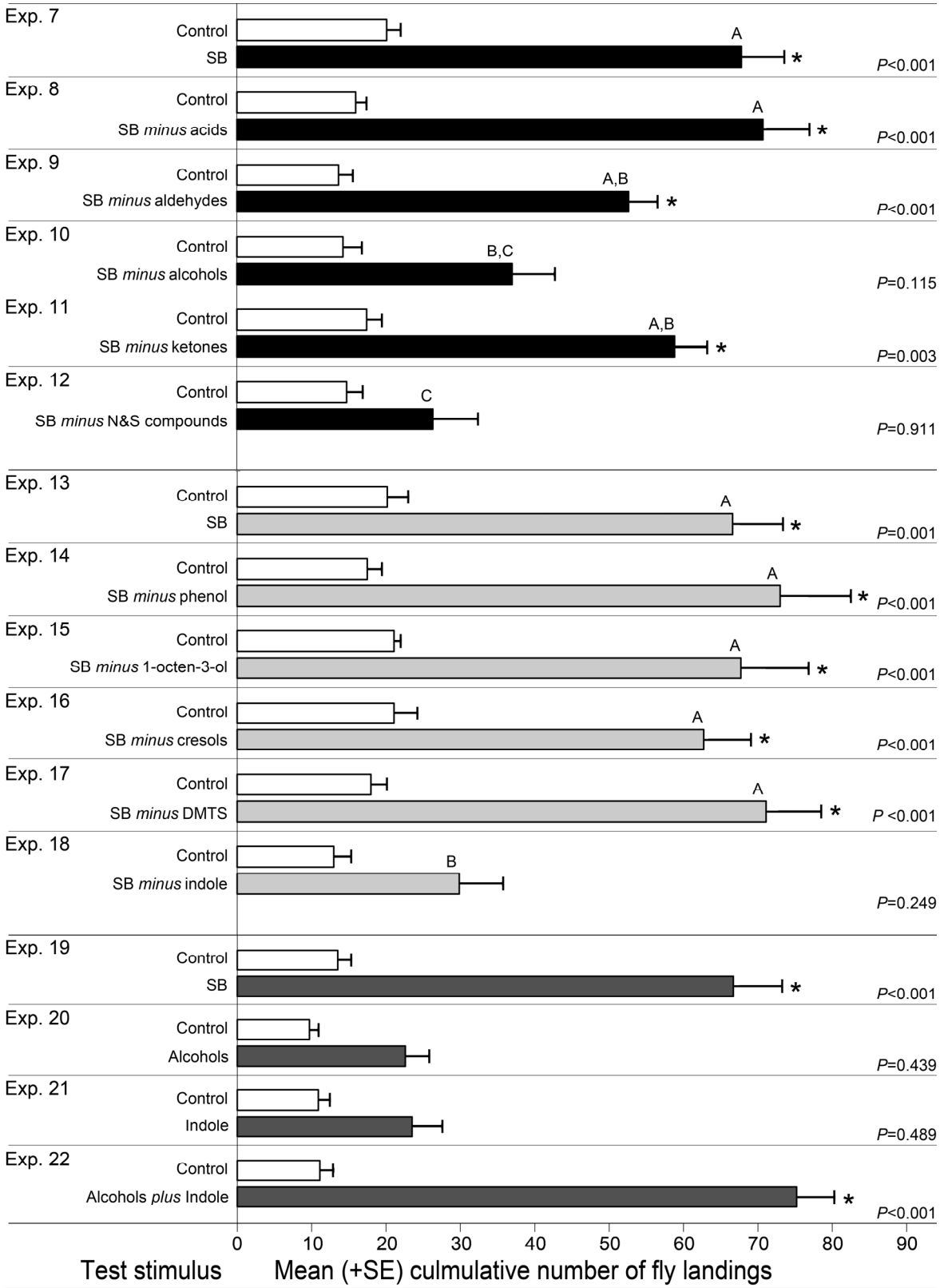
Exp. 7
Control
SB
A
*
P<0.001
Exp. 8
Control
SB minus acids
A
*
P<0.001
Exp. 9
Control
SB minus aldehydes
A,B
*
P<0.001
Exp. 10
Control
SB minus alcohols
B,C
P=0.115
Exp. 11
Control
SB minus ketones
A,B
*
P=0.003
Exp. 12
Control
SB minus N&S compounds
C
P=0.911
Exp. 13
Control
SB
A
*
P=0.001
Exp. 14
Control
SB minus phenol
A
*
P<0.001
Exp. 15
Control
SB minus 1-octen-3-ol
A
*
P<0.001
Exp. 16
Control
SB minus cresols
A
*
P<0.001
Exp. 17
Control
SB minus DMTS
A
*
P <0.001
Exp. 18
Control
SB minus indole
B
P=0.249
Exp. 19
Control
SB
*
P<0.001
Exp. 20
Control
Alcohols
P=0.439
Exp. 21
Control
Indole
P=0.489
Exp. 22
Control
Alcohols plus Indole
*
P<0.001
0
10
20
30
40
50
60
70
80
90
Test stimulus
Mean (+SE) culmulative number of fly landings

Figure 3.5. **Mean (+ SE) cumulative number of alighting responses by young females of *Lucilia sericata* in experiments 7-22 (n = 10 each) on paired, cheesecloth-covered Solo cups (see Fig. 1a) containing filter paper impregnated with (*i*) a synthetic blend (SB; Table 1) of the 17 components that elicited antennal responses in headspace volatile extracts of canine feces (see Fig. 4), (*ii*) partial blends of these components, or (*iii*) select key components like indole and alcohols (phenol, 1-octen-3-ol, *m-/p*-cresol). Filter paper impregnated with the corresponding amount of pentane served as the control stimulus. In each experiment, an asterix (*) denotes a treatment stimulus that received significantly more than 65% of the flies' alighting responses (one-tailed t-test: $P<0.001$). Within experiments 7-12, 13-18, and 19-22, bars with different letters indicate significant differences in the number of alighting responses by young female flies (Tukey's HSD: $P<0.05$).

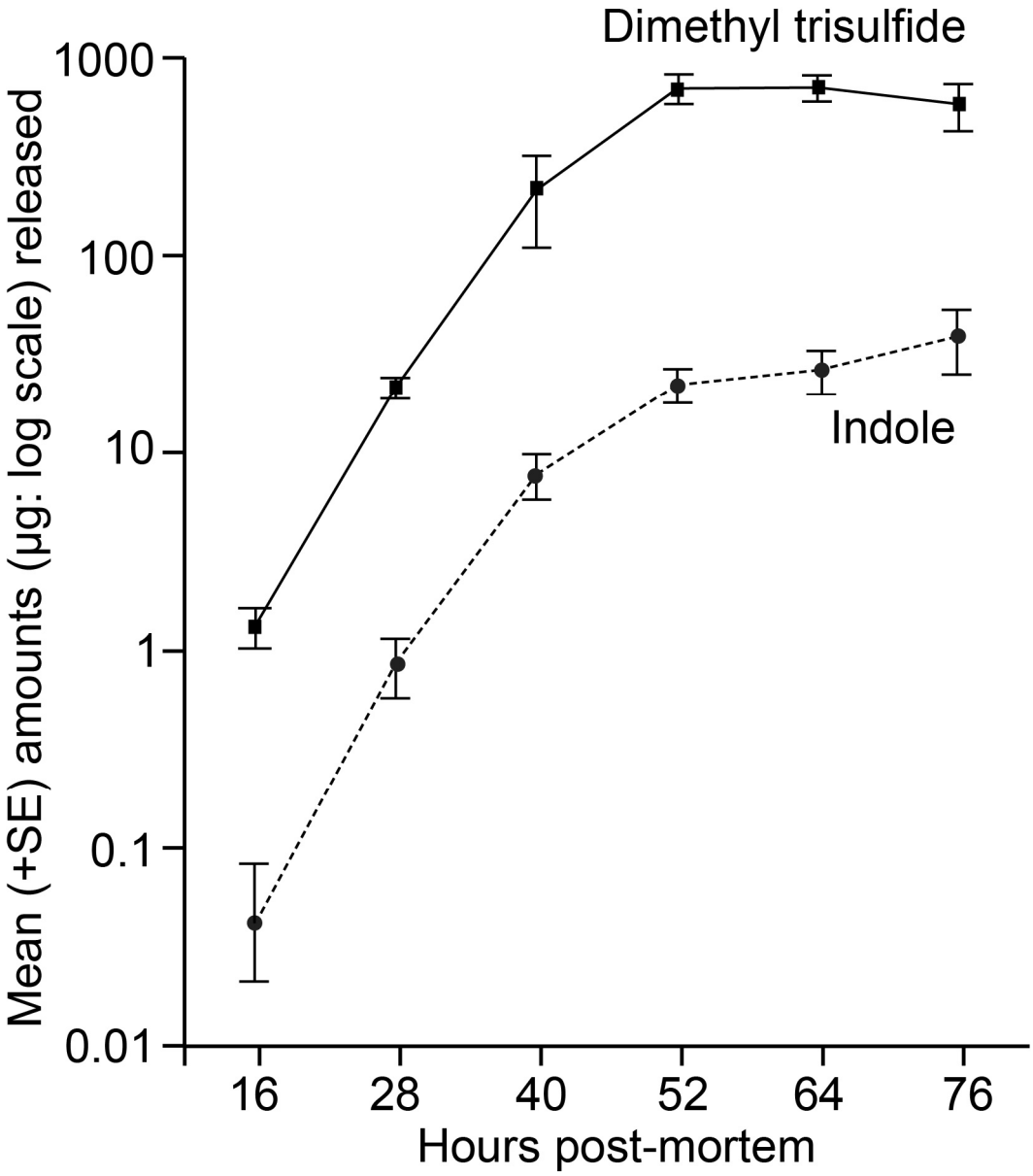

Figure 3.6. **Mean (± SE) amount of dimethyl trisulfide (DMTS) and indole released from 10 CO_2-euthanized mice (n = 3) during six time intervals *post mortem*. Note that only trace amounts of indole are present in the early phase of decomposition.**

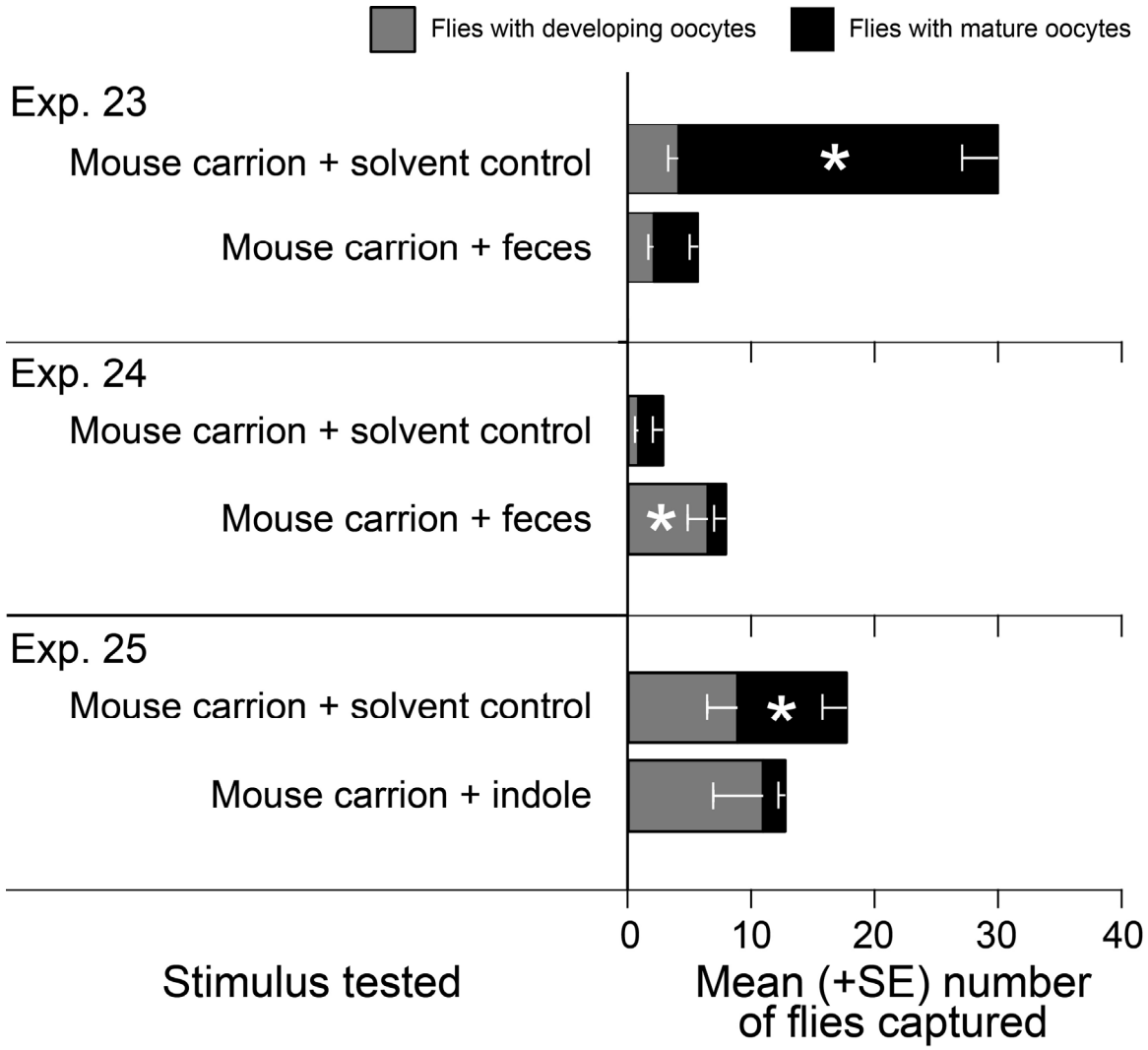

Figure 3.7. **Mean (± SE) number of *Lucilia sericata* females captured in paired Oak Stump traps (see Fig. 1b) baited with (*i*) one mouse carcass alone or in combination with fresh canine feces (20 g) (laboratory experiment 23: 24-h-old mouse carcass; field experiment 24: fresh mouse carcass), or (*ii*) one fresh mouse carcass alone or in combination with indole (100 µg) dissolved in ether and applied to a cotton ball (field experiment 25). In each experiment, an asterisk (*) denotes the stimulus that attracted significantly more flies of a particular reproductive stage (Wilcoxon signed rank test; *P*< 0.05).**

Chapter 4.

Flashy mate recognition cues of the common green bottle fly *Lucilia sericata*?

Eichorn, C., M. Hrabar, B.S. Brodie, A. Blake, E. Van Ryn, D. Altshueler, and G. Gries; a manuscript with additional data is currently in preparation.

As the third author, I made substantial contributions to the conception and design of the work, interpretation of the data, and drafting the manuscript.

4.1. Abstract

Locating mates is usually a hierarchical process in which visual cues merely reinforce long-range semiochemical signals. Here, we show that males of the common green bottle fly, *Lucilia sericata* (Diptera: Calliphoridae), respond to long-range visual mate location or recognition cues. We present data revealing that (1) wing movement of females is a (visual) mate location factor, (2) wings are thin-film reflectors that produce light flashes during movement, (3) light flashes are absent or reduced under diffuse or overcast light, and (4) light flashes differ in frequency between males and females. While wings of *L. sericata* also produce stable structural colours (= wing interference patterns), UV- and polarized-light reflections, these specific optic effects per se do not appear to serve as mate recognition cues. Instead, there is mounting evidence for a new mating system in flies, where the frequency of light flashes reflected off moving female wings allows males to locate and recognize prospective mates.

4.2. Introduction

General introduction

Sexually reproducing animals face the challenge of attracting or locating appropriate mates (Triplehorn & Johnson, 2005). Communication signals are shaped by natural selection to carry specific meanings (Saleh et al. 2007) and are a highly effective tool for increasing the rate of male-female encounters, but in the absence of specific signals, mate-seeking individuals may instead rely on cues (Bradbury & Vehrencamp, 1998). Unlike signals, cues are not sent intentionally by the signaler to an intended receiver, but instead represent some attributes of the signaler that the receiver exploits to obtain information (Seeley, 1989). In addition to providing information about the sender, some aspects of a cue may function to improve cue detection or transmission (Candolin, 2003; Schultz & Fincke, 2009).

Evidence supporting that blow flies use visual cues for mate location

Although dipterans, blow flies (Calliphoridae) share many of the visual capabilities attributed to specific families in the Lepidoptera (Sweeney et al., 2003; Papke et al., 2007), Hymenoptera (Sheehan et al., 2014) and Odonata (Gorb, 1998; Schultz & Fincke, 2009) that reportedly rely on visual mate recognition signals and cues. In common with insects that use visual cues, blow flies have large, conspicuous eyes with >5,000 ommatidia (Sukontason et al., 2008). Large size and numbers of ommatidia effectively improve the visual acuity of compound eyes (Land, 1997). Blow flies are tri-chromatic (Fukushi, 1989), with absorption maxima in the ultraviolet range (about 344 nm), blue range (443), and green range (about 490 nm) (Meffert & Smola, 1976; Smola & Meffert, 1979), and according to behavioural bioassays blow flies can discern between colours (Fukushi, 1985). Additionally, UV photoreceptors in the dorsal rim of blow fly eyes are sensitive to polarized light (Briscoe & Chittka, 2001).

Fly species are generally thought to be highly visual (Lunau, 2014). In flies, reliance on vision compliments their advanced flying capability which requires vision to gauge distance traveled and to avoid collisions (Lunau, 2014). Blow flies also use visual cues when foraging (Wall & Fisher, 2001; Gomes et al., 2007) or seeking oviposition sites

(Brodie et al., 2014a). Blow flies are known not to have sex pheromones or sexually dimorphic cuticular hydrocarbons (Stoffolano et al., 1997), but to be highly visual (Hardie et al., 2012) and to hilltop (Merz. 2000), suggesting that blow flies may rely on vision as the dominant sensory modality to locate mates. Boeddeker et al. (2003) support this concept concluding that male flies rely on vision to pursue and catch prospective mates in high-speed acrobatic chases. Our own bioassay data (Eichorn & Brodie, unpubl.) suggest that males of the common green bottle fly, Lucilia sericata, use visual rather than olfactory cues to recognize prospective mates.

Do flashing cues from moving wings help L. sericata males detect mates?

Flashing cues produced by moving wings coupled with species- and sex-specific reflectance of polarized light, structural colours, and iridescence are thought to mediate long-range detection of potential mates in highly visual flying insects (Sweeney et al., 2003; Schultz & Fincke, 2009; White et al., 2014). Wings as thin-film reflectors, as reported for various insect species (Sweeney et al., 2003; Schultz & Fincke, 2009; Shevtsova et al., 2010; White et al., 2014), reflect light in a highly directional way by optical interference (Vukusic et al., 1999). Thus, as a wing rotates during flight relative to the position of the sun, reflections flash on and off (Schultz & Fincke, 2009). In comparison to static cues, flashing cues enhance contrast and conspicuousness, and thus facilitate improved cue conveyance (Schultz & Fincke, 2009) and sensory reception (von Grünau et al., 1999), resulting in long-range cue visibility. For example, tropical *Morpho* butterflies are reportedly visible from low-flying aircraft (Silberglied, 1984). Iridescent flashes produced by wings of butterflies and damselflies are thought to aid in mate location and recognition (Sweeney et al., 2003; Schultz & Fincke, 2009). Courting males of the common egg fly, *Hypolimnas bolina* (L.) (Lepidoptera: Nymphalidae), position themselves relative to females such that they enhance the flashing effect of directional UV patches on their wings, a behavior that implicates flashes as cues which improved information conveyance (White et al., 2014). Interestingly, on overcast days when otherwise direct illumination from the sun becomes diffuse (Endler, 1992, 1993) and reduces any flash effects (White et al., 2014), blow flies have a lower propensity to mate (Rutowski, 1992).

We predict [**Hypothesis (H) 1**] that male *L. sericata* discern between mates and males, and that wing movement is a mate recognition factor for male flies. Drawing on previous findings that the wings of small flies consist of two layers of chitin fused together into a single membrane that acts as a thin-film reflector (Nachtigall, 2011), we also predict (**H2**) that wings of *L. sericata* are thin-film reflectors of chitinous structure that produce interference effects and light flashes during movement. Given that *L. sericata* appears reluctant to mate on overcast days (personal observation), we further predict (**H3**) that flashing cues are absent or reduced under diffuse or overcast light.

Do specific optic effects produced by moving L. sericata wings serve as mate recognition cues?

Wings as thin-film reflectors of various forms and with distinct micro-structures produce a range of chromatic and achromatic colour effects (Kinoshita et al., 2008), UV reflections (Ghiradel et al., 1972; Vukusic & Sambles, 2003) and polarized-light reflections (Vukusic et al., 2000) that insects might use in mate recognition. For example, both males and females of the checkered white butterfly, *Pieris protodice* (Boisduval and Leconte) (Lepidoptera: Pieridae), recognize prospective mates based on sexually dimorphic UV reflectance off wings (Rutowski, 1981), whereas species within the butterfly genus *Heliconius* (Lepidoptera) rely on polarized-light reflections off wings for mate recognition (Sweeney et al., 2003). Similarly, the wings of small flies produce stable structural colours termed wing interference patterns (WIPs), which have been hypothesized to function in mate recognition (Shevtsova et al., 2011). We predict (**H4**) that *L. sericata* wings produce WIPs, UV, or polarized-light reflections that singly or in combination provide mate recognition cues.

Does the frequency of light flashes serve as a mate recognition cue?

The visual system of blow flies operates at a remarkable processing speed (Lunau, 2014), thus facilitating detection and recognition of images that move at high speed (Matthews & Matthews, 2010). *Lucilia sericata* (*Diptera: Calliphoridae),* e. g., can resolve >300 frames per second (Ruck, 1961). Furthermore, the fritillary butterfly *Argynnis paphia* (Lepidoptera: Nymphalidae) shows preference for higher flash frequencies (Magnus, 1958), suggesting that at least some insect species can discern among different flash frequencies. Considering that male and female *L. sericata* differ

greatly in body size (personal observation), it is conceivable that they also differ in wing beat frequency. We predict (**H5**) that *L. sericata* has a gender-specific frequency of wing flashes that alone or in combination with other optic effects of wings mediates mate recognition.

4.3. Materials and methods

4.3.1. Source of flies

Blow flies, *L. sericata*, were reared in the insectary of Simon Fraser University, starting a new colony with field-collected wild flies. To ensure that virgin flies were tested in various experiments, we kept eclosed flies without food for 24 h, thereby reducing the likelihood of mating (Stoffolano et al. 1995; Stone et al. 2009). We then separated cold-sedated flies by sex (Thomas, 1993) and kept them in groups of 50 males or 50 females in separate wire mesh cages (61 × 61 × 61 cm; BioQuip®, Compton, CA, USA). We maintained these cages under a L16:D8 photoperiod, a 30-40% relative humidity, and a temperature of 23-25 °C. We provisioned flies with water, milk powder, sugar and liver *ad libitum* and bioassayed them when they were 5 to 7 days old, the age range where flies are sexually most active (personal observation; Thomas, 1993).

4.3.2. General design of two-choice behavioural bioassays

For each experimental replicate, we placed a cage with 50 virgin male response flies (RFs) under a full spectrum light source [two horizontal mercury lamps: Philips, plant & aquarium (40 W); Sylvania, Daylight deluxe (40W)]. After a 30-min acclimation period, we removed the food and offered the RF males a choice between two live stimulus flies (SFs) that we had mounted with super glue (Gorilla Glue®, Cincinnati, OH, USA) 7 cm apart from one another on an aluminum T-bar (Fig. 1a) and rotated between experiments to remove position effects. We mounted SFs on their abdominal ventrum with their legs dangling to induce a wing-fanning response. Exposing the two SFs to CO_2 for 30 s facilitated their mounting and conglutinating or spray-painting of their wings (see below). To minimize light reflections from the experimental setting that might cancel out potential interference effects of the wings (Shevtsova et al., 2011), we covered the metal cage floor

and T-bar stand with SunWorks® black construction paper (Pacon Corporation, Appleton, WI, USA) and with black velvet (Dressew supply, Vancouver, BC, Canada), respectively.

In each experimental replicate, we gave RF males 20 or 40 min to respond to mounted SFs, and scored four behavioural responses of RF males: (1) the number of "landings" on either SF; (2) the number of (attempted) copulations with a SF; (3) the SF sought first for a copulation (attempt); and (4) the length of time they spent in copula, or attempting to copulate, with a SF. Here we define landing by a male RF as alighting either directly on a SF or on the T-bar and then making physical contact with a SF. We note a copulation attempt when a male RF attempted for ≥30 s to copulate with a SF, in the process bending his abdomen around the SF and extending his adeagus.

Do flashing cues from moving wings help L. sericata males detect potential mates?

H1: (a) males discern between mates and males, and (b) wing movement is a mate recognition factor

To test H1a (Table 1), we followed the general bioassay design described above. We glued a male and a female SF on the horizontal arm of the T-bar (Fig. 1a), which we introduced into a bioassay cage with 50 RF males, scoring four behavioural responses of RF males (see above) for 20 min in each replicate.

To test H1b (Table 1), we mounted both SF females on the T-bar (see above) and applied a small amount of glue (Gorilla Glue®, Cincinnati, OH, USA) to the wing base of the treatment SF female thus immobilizing her wings, and the same amount of glue to the abdomen of the control SF female. We then introduced the T-bar into the bioassay cage with 50 RF males, scoring four behavioural responses of RF males (see above) for 40 min in each replicate.

H2: (a) wings are thin-film reflectors that (b) produce light flashes during movement

We tested H2a (Table 1) using transmission electron microscopy (TEM). From freshly killed male and female flies we removed the wings, treated them for 1 h with a primary fix (2.5% glutaraldehyde in 0.1M Na cacodylate buffer), replaced the fixative with

a buffer solution (0.1M Na cacodylate buffer), and then stored the wings in the buffer solution overnight at 4 °C. The following day, we treated wings with a secondary fixative (0.1M Na cacodylate buffer) and placed them in a Pelco Laboratory microwave/vacuum for two 2-min-on 2-min-off cycles. Following a distilled water wash, we dehydrated the wings in a graded series of ethanol and at each step microwaved the samples for 30 s to ensure the fixative was fully incorporated. Finally, we embedded the wings in a graded series of Spurs Resin, microwaving the sample for 3 min at each step. We sectioned samples using an ultra-microtome, stained them in sequence with lead citrate and 2% aq uranyl acetate, and then imaged them with a Hitachi H-760 TEM, operating at 80 kV and capturing images with a AMT XR50 camera.

To test H2b (Table 1), we used high-speed video to record the wing movement of abdomen-mounted male and female flies (Fig. 1b). To capture slow motion images, we used a Phantom Miro 4 camera (Vision Research, Wayne, NJ, USA), recording at 15,000 fps, a 512 × 512 pixel resolution, and a 20-μs exposure time. To illuminate the mounted fly, we used a white 100-watt LED (6500K; Zongshan Ltd., Guangdong, China) mounted to a computer CPU heat sink for cooling (Thermaltake Heatpipe, Thermaltake Technology Co. Ltd, Taipei, Taiwan), and powered via a 32V 5A stabilized, adjustable power supply (Gopher Technologies, Yantian, Fenggang, Dongguan, Guangdong, China).

H3: wing flashing cues are absent or reduced under diffuse light

To test H3 (Table 1), we used the same high-speed video technology as described for testing H2b except that the mounted fly was exposed to diffuse instead of direct light. The fly was placed inside a ping pong ball “diffuser” (Fig. 1c) and illuminated by four cool white 100-watt LEDs (see above).

Do specific optic effects produced by moving wings serve as sex recognition cues?

H4: (a) wings produce optic effects (WIPs, UV-, or polarized-light reflections) that (b) singly or in combination provide mate recognition cues

To study WIPs on wings of males and females (H4a; Table 1), we removed wings, placed them on black velvet inside a spherical (ping pong ball) diffuser (Fig. 1c), and illuminated the diffuser with three 100-watt LEDs. We captured images with a Canon 5D

MKII camera (Canon USA Inc., Mellville, New York, USA) fitted with a 100-mm macro lens (EF 100L; Canon USA Inc., Mellville, New York, USA), using an exposure time of 15 s at f16.

To determine whether UV or or polarized-light reflections are sex-specific (H4a; Table 1), we used polarimetry to image the wings of male and female flies. To obtain these images, we placed wings dorsal side up and angled at 45° to a Hortilux - Blue™ daylight metal halide lamp (MT400D/BUD/HTL-BLUE, EYE Hortilux, USA) mounted above the wing (Fig. 2b). We took the images with a custom-designed camera system (purchased from Dr. Klaus Schmitt, Wienheim, Germany; uvir.eu), consisting of an Olympus EPM1 camera and a 35-mm lens modified for enhanced UV sensitivity. We aligned the optical axis of the camera to point strait toward the mounted wing, at a 90° angle to the light source (Fig. 2b).

We took two sets of four photographs of each wing, using an ultra broadband linear polarizing filter (68-751, Edmund Optics, USA) that we manually positioned so that the maximum axis of transmission was oriented at 0°, 45°, 90°, and 135° from vertical. One set of four photographs captured the image in the UV range, using a U filter (Baader-U filter, Baader Planetarium, DE). The other set of four photographs captured the image in the human-visible range using a UV/IR Cut Filter (2 "Baader UV/IR Cut/L-Filter, Baader Planetarium, DE (Fig. 1c). We saved photographs as RAW images and developed them to preserve linearity in light intensity. We split the visual images into red (R), blue (B), and green (G) colour channels, and processed the UV images as grayscale images (Fig. 2c). We aligned photographs in ImageJ (http://imagej.nih.gov/ij/index.html), using the TurboReg plugin (http://bigwww.epfl.ch/thevenaz/turboreg/) and a custom macro. We analyzed the single-colour channel images using stokes vectors via another custom ImageJ macro that created images displaying the intensity (ı) as well as the degree (ρ) and angle of polarization (α) for each colour channel (Fig. 2c).

To test the importance of WIPs, UV- or polarized-light reflections on the response of males in behavioural bioassays (H4b; Table 1), we mounted two SF females to the T-bar (Fig. 1a), and with stencils airbrushed (Hohmi Dash Y3D airbrush, Holbein, Japan) black acrylic paint (high flow acrylic paint # 8524, Golden Artists Colours Inc, USA) to the

most reflective region of the wing (including the WIP) of the treatment SF female and to the wing base of the control SF female (Fig. 2a). We then introduced the T-bar into the bioassay cage with 50 RF males, scoring four behavioural responses of RF males (see general design of two-choice behavioural bioassays) for 20 min in each replicate.

Does the frequency of light flashing cues serve as sex recognition cues?

H5: *L. sericata* has a gender-specific frequency of wing flashes that alone or in combination with other optic effects of wings mediates mate recognition

To test H5 (Table 1), we mounted a 3- to 7-day-old male or female fly on the T-bar (Fig. 1a) and recorded its wing beat frequency. Recordings took place in a sound-dampened room, using a Sony FV 120 condenser microphone (Sony of Canada Ltd., Toronto, Ontario M2H 3R6, Canada) positioned 1.5 cm apart from an abdomen-mounted, wing-fanning fly. We converted recorded wing beat sounds to a WAV file, using the software program Raven lite 1.0 on a MDG Intel ® Core ™2 Duo CPU T5250 @ 1.5 GHz computer equipped with RealTech High Definition Audio at a sampling rate of 44.1 kHz. We analyzed recordings for waveform, frequency, and time-frequency sound intensity (sonogram), with Raven Lite 1.0 using 2048 lines for the Fourier transform. To determine the mean wing beat frequency of male flies and of female flies, we ran a 1-min recording session for each fly, averaged the dominant frequency of all wing-fanning bouts for each fly, and then averaged the dominant frequency across males and females.

4.3.3. Statistical analyses

In each of the two-choice (mounted treatment fly *versus* mounted control fly) experiments, we compared the mean proportion of males that chose to copulate or attempted to copulate with a treatment fly first to a hypothesized mean of 0.5 using a chi-squared test. We also compared the mean proportion of male landings, and the mean proportion of time males spent in copula or attempting to copulate to a hypothesized mean of 0.5 using a two-tailed Wilcoxon signed-rank test, predicting that if males preferred the treatment or the control fly, the proportion would be >0.5. We also compared the difference between the number of mating attempts with the treatment fly to that with the control fly (treatment minus control) to a hypothesized mean of 0, using a two-tailed Wilcoxon signed-rank test. We did not analyze data from trials where RF males

did not attempt to mate with either stimulus fly. We compared the mean wing beat frequency of males and females using a two tailed t-test. For all statistical analyses we used JMP 10® (SAS Institute Inc.) for Mac® (Apple Inc., Cupertino, CA, USA).

4.4. Results

Do flashing cues from moving wings help L. sericata males detect potential mates?

H1: (a) males discern between mates and males, and (b) wing movement is a mate recognition factor

In the two-choice bioassays that tested H1a (Table 1), SF females did not receive significantly more than 50% of the alighting responses by RF males (Fig. 3a). However, SF females were invariably sought first for copulatory attempts by RF males (Fig. 3b), received all copulatory attempts by RF males (Fig. 3c), and induced RF males to spend all their time copulating or attempting to copulate with them (Fig. 3d). Combined, these results indicate that *L. sericata* males distinguished between mates and males.

In the two-choice bioassays that tested H1b (Table 1), SF females with fully functional wings did not receive significantly more than 50% of the alighting responses by RF males (Fig. 4a). However, SF females with fully functional wings were invariably sought first for copulatory attempts by RF males (Fig. 4b), induced all copulatory attempts by RF males (Fig. 4c), and induced RF males to spend all their time copulating or attempting to copulate with them (Fig. 4d). Combined these results indicate that wing movement is a mate recognition factor used by *L. sericata* males to distinguish between mates and males.

H2: (a) wings are thin-film reflectors that (b) produce light flashes during movement

Transmission electron micrographs (H2a; Table 1) of male and female *L. sericata* wings (Fig. 5ab) revealed that the dorsal and ventral membranes are fused forming a single thin layer, as shown for *Drosophila* spp. (Shevtsova et al., 2011). These results indicate (a) that wings of *L. sericata* could act as thin-film reflectors producing highly

directional optic effects, and (b) that these optic effects do not appear to be gender-specific.

Testing H2b under direct light (Table 1) via high-speed video recordings (Fig. 1b) revealed that the wing movement of abdomen-mounted female flies caused qualitative changes in light intensity reflected off wings during a wing beat cycle (Fig. 6, a-d).

H3: wing flashing cues are absent or reduced under diffuse light

Testing H3 under diffuse light (Table 1) via high-speed video recording (Fig. 1c) revealed that the wing movement of abdomen-mounted female flies failed to cause qualitative changes in light intensity reflected off wings during a wing beat cycle (Fig. 6e-h).

Do specific optic effects produced by moving wings serve as sex recognition cues?

H4: (a) wings produce optic effects (WIPs, UV-, or polarized-light reflections) that (b) singly or in combination provide mate recognition cues

In photographs taken to address H4a, wings of females and males revealed only subtle gender differences in WIP colour patterns (Fig. 7). The wing tip of females has a magenta spot, whereas the same region is green in the wings of males.

Polarimetry images of male and female wings analyzed using IMAGE-J to obtain mean values of intensity and degree and angle of polarization (Fig. 8) revealed (*i*) that both male and female wings reflect light in the visible and UV range [intensity (ı): red ≈ 8%, green ≈ 13%, blue ≈10%, UV ≈ 0.35%], and (*ii*) that across all colour channels the reflected light is about 60% polarized (ρ), at an angle (α) of 90°. Polarimetry images of male and female wings did not reveal any sexual dimorphisms in the intensity (ı), degree (ρ), or angle (α) of polarization. Intensity measurements are relative to the number of photons that would saturate the camera sensor (~7900 photons) and are not an absolute value of reflectance.

In the two-choice behavioural bioassays that tested H4b, control SF females with their wing base rather than the most reflective region (MRR) of their wing spray-painted

(Fig. 2a) did not receive significantly more than 50% of the alighting responses by RF males (Fig. 9a). Similarly, control SF females did not induce significantly more than 50% of the copulatory attempts by RF males (Fig. 9c). However, control SF females were sought first by RF males for copulatory attempts significantly more often than treatment SF females that had the MRR of their wings spray-painted (Fig. 9b). Also, control SF females induced RF males to spend significantly more time copulating or attempting to copulate with them than with treatment SF females (Fig. 9d).

Does the frequency of light flashing cues serve as mate recognition cues?

H5: *L. sericata* has a gender-specific frequency of wing flashes that alone or in combination with other optic effects of wings mediates mate recognition

The dominant wing beat frequency of females (mean ± SE: 197 ± 4.93 Hz) was on average 12.15 Hz higher than that of males (185 ± 7.01Hz), but this difference was not statistically significant ($t = 1.38$, $df = 13$, $p = 0.19$).

4.5. Discussion

Our data support the hypotheses that (1) wing movement is a (visual) mate recognition factor that *L. sericata* males use to distinguish between mates and males at a distance, (2) *L. sericata* wings are thin-film reflectors that produce light flashes during movement, and (3,4) light flashes are absent or reduced under diffuse or overcast light and (although not statistically significant) differ in frequency between males and females. We conclude that wings of *L. sericata* do produce WIPs, UV- or polarized-light reflections but argue, based on the lack of differences between sexes, that these specific optic effects *per se* do not provide mate recognition cues. Instead, based on preliminary data, we predict that *L. sericata* has a gender-specific frequency of wing flashes, which may help males recognize prospective mates. This prediction would have to be tested experimentally.

To test whether *L. sericata* males distinguish between mates and males (H1a, Table 1), we gave virgin RF males a choice between a SF male and a SF female mounted on a T-bar (Fig. 1a). The fact that RF males attempted to copulate only with the

SF female in each trial (Fig. 3c) indicates that *L. sericata* males distinguish between males and mates. Wing movement appears to be a mate recognition cue. This became apparent when we gave virgin RF males a choice between a mounted female that could wing fan, and a mounted female with her wings experimentally immobilized (H1b, see Table 1). In every trial, RF males attempted to mate only with females that could wing fan (Fig. 4c). The win fanning cue most likely functions at long range because males hill-top (Merz 2000), sit perched in their territories, and pursue females of rival males flying into their territory.

Although the preference of males for females with functional wings could be attributed to visual, chemosensory, thermal, or auditory cues associated with moving wings, we argue that visual cues play a primary role. Wing fanning can function to disseminate sex attractant pheromones towards potential mates (Shelly & Kaneshiro, 199; Canale et al., 2013) but there is no evidence for specific sex attractant or contact mate recognition pheromones in blow flies (Benziane & Campan, 1993; Stoffolano et al., 1997; Brodie et al., 2014b). Flying or wing fanning generates heat (Heinrich, 1971; May, 1995) and corresponding infrared radiation, which could function as a mate-location cue. Some insects such as fire beetles (Evans 1964; Schmitz et al. 1997, 2000, 2008) and seed bugs (Campbell et al., 2002; Takács et al., 2009) are known or implied to possess infrared receptors, and heat receptors are not "designed" to process directional information. Finally, moving wings produce wing beat sounds that could be exploited as an auditory mate-location cue by males. Mosquito males, e.g., locate swarming females by sensing their wing beat frequency with sound receptors, the Johnston's organ, on their antennae (Roth, 1948; Gibson & Russell, 2006). Although antennal hearing is ubiquitous across higher dipteran taxa (Robert & Gopfert, 2002), mosquitoes exhibit specific adaptations for locating mates via auditory cues that are not necessarily found in blow flies. Mosquito males have exceptionally sensitive Johnston's organs (Mason et al., 2001) and females have specialized structures at the wing base that help produce sound (Matthews & Matthews, 2010). Such specialization may have evolved as an adaptation to mate-foraging at dawn and dusk when visual cues become less apparent. Blow flies, in contrast, occupy open habitats with lots of direct light (Erzinçlioglu, 1996), seek mates mostly in full sun light (personal observation), and thus could rely exclusively on visual mate location cues. Furthermore, the large and elaborate mosquito antennae house

auditory, thermal, and contact-chemo receptors (Boo, 1980), whereas antennae of blow flies are too small to play a functional role in contact chemo recognition of potential mates (Lunau, 2014), and possibly to sense other mate recognition signals. However, in comparison to most other insects, blow flies have some of the most elaborate visual systems (Lunau, 2014), with specialized “bright zones” that probably function to aid in the location of mates (Vanhateren et al., 1989; Straw et al., 2006; Sukontason et al., 2008b).

To study the visual cues associated with wings *of L. sericata* males and females, we took transmission electron micrographs (TEMs) of wing sections. These TEMs revealed that *L. sericata* wings are thin-film reflectors (Fig. 5; Vukusic et al., 1999; Shevtsova et al., 2011) but failed to reveal sex-specific differences other than width. Filming wing movement of abdomen-mounted females by high speed videography documented qualitative changes in light intensity reflected off wings during a wing beat cycle (Fig. 6a-d), generated by the thin-film-reflector effect. Put more simply, the moving wings of free flying or abdomen-mounted *L. sericata* females produce regular flashes of light. At a distance, these flashes may appear to mate-seeking males as orbs of light flashing on and off, analogous to flashing light signals produced by bioluminescent fireflies (Llyod, 1983; Bradbury & Vehrencamp, 2011). That *L. sericata* males do not rigorously pursue females on overcast days, when light flashes off wings are absent or reduced (Endler, 1992; Endler, 1993; White et al., 2014) (Fig. 6e-h), and thus when prospective mates are less conspicuous, support the interpretation that flashing lights associated with moving wings could be mate-location cues in *L. sericata*, as proposed for various taxa of insects (Magnus, 1958; Sweeney et al., 2003; Schultz & Fincke, 2009; White et al., 2014).

Anticipating sex-specific optical characteristics of light reflections off male and female *L. sericata* wings, we applied various experimental techniques to study these characteristics. While wings of males and females reflect UV and linearly polarized light (Fig. 8), there were no obvious sex-specific differences in these light characteristics that could possibly mediate mate recognition. Because there were subtle sex-specific differences in the colour and arrangement of WIPs (Fig. 7) produced by the thin-film structure of wings (Fig. 5ab), we decided to test the effect of WIPs for mate recognition. we spray-painted the most reflective wing region to occlude WIPs (Fig. 2a), and indeed

noticed fewer copulations of males with such wing-painted females, suggesting that WIPs may matter in mate recognition. However, when we filmed the wing movement of wing-painted females in slow motion, it became apparent that the paint coat on the wings severely altered wing movements that, in turn, may have rendered such females less appealing to males. To determine whether the subtle sex-specific differences in WIPs contribute to mate recognition in *L. sericata,* an alternative bioassay will need to be designed.

Hypothesizing that male and female *L. sericata* differ in wing beat frequency (WBF), and thus the number of light reflections off wings per second, we recorded the wing beat sound of male and female flies mounted in the same way as in behavioural bioassays (Fig. 1a). While the first harmonic of WBF sound of males and females differed by 12 Hz, on average, this difference was not statistically significant possibly due to the uneven age of flies we used in our recordings, an insufficient number of recordings, or placement of the microphone in front of the flies. However, a separate study with the microphone lateral to flies reports that the second harmonic of WBF sound of *L. sericata* males (390 Hz) and females (377 Hz) differed significantly (Sueur et al., 2005). Within this frequency range, *L. sericata* males are likely to resolve the light flashes reflected off wings of flying conspecifics. The flicker fusion threshold (= frequency at which a rapidly blinking light is perceived as continuous (Bradbury & Vehrencamp, 2011)) of flies has been measured at >300 Hz and might approach 400 Hz or 500 Hz when a fly is at optimal temperature (Tatler et al., 2000). In light of the flies' ability to resolve extremely fast visual cues, it seems conceivable that the rate of light flashes produced by moving wings of *L. sericata* females serves males as a mate recognition cue. This prediction would need to be tested in bioassays with males responding to female fly models that are coupled with artificial light flashes at male- or female-specific wing beat frequencies. Flashes of light produced by moving insect wings have been hypothesized, but never tested experimentally, to contribute to sex recognition (Sweeney et al., 2003; Schultz & Fincke, 2009) in various insect species.

In conclusion, *L. sericata* males appear to rely on visual rather than semiochemical cues to locate and recognize potential mates. While there is evidence indicating that gravid *L. sericata* females exploit both visual and olfactory cues associated

with oviposition sites (Brodie et al., 2014a), visual cues in the context of mate recognition had never been studied in *L. sericata*. If the sex-specific frequency of flashing lights reflected off wings is indeed a mate recognition cue, this would explain why aggregation or sex pheromones are not essential for sexual communication in *L. sericata,* and thus why they may not exist and could not be found (Benziane & Campan, 1993; Stoffolano et al., 1997; Brodie et al., 2014b).

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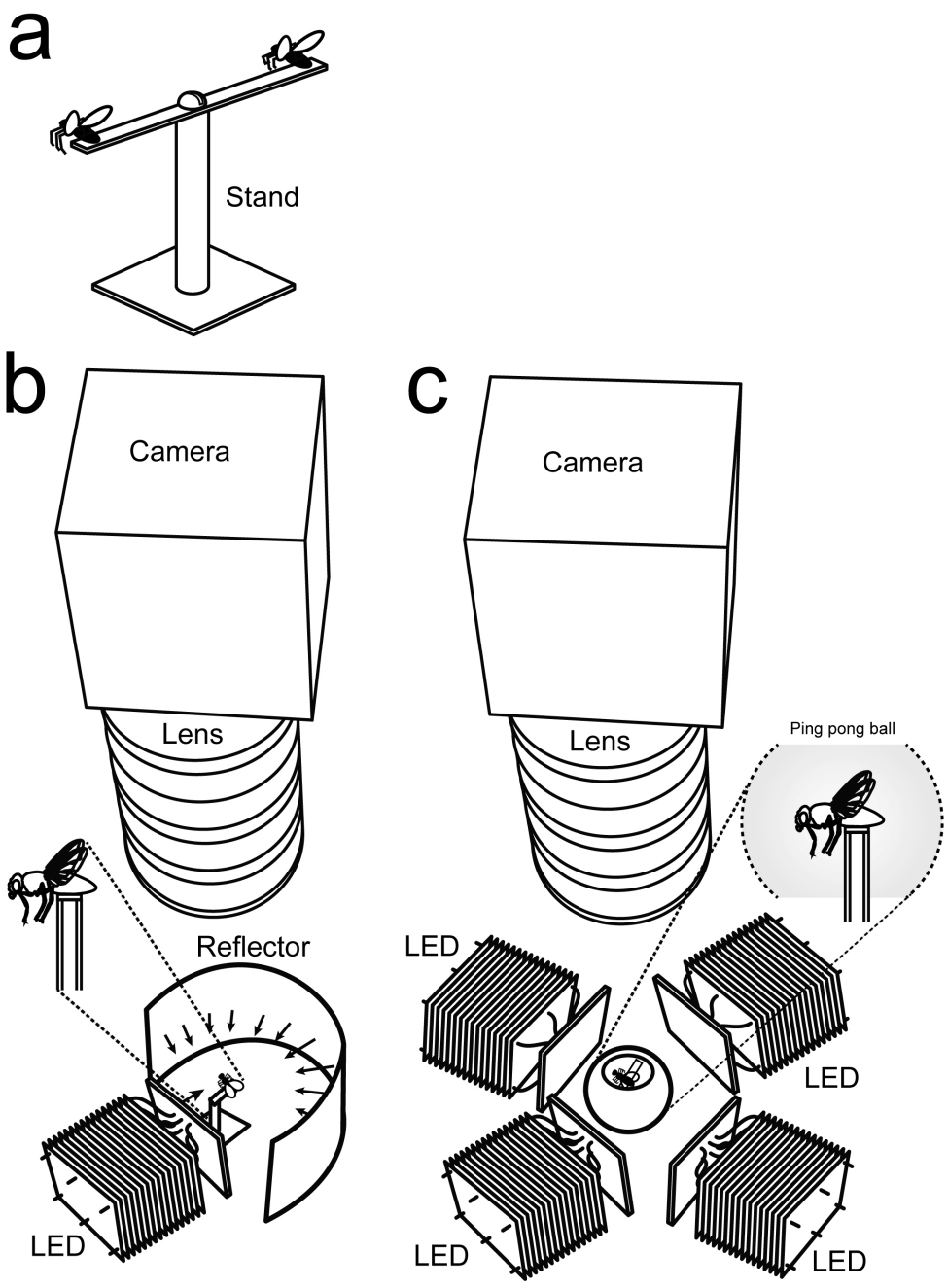
Table 4.1. List of (1) research questions addressing visual mate recognition cues of *Lucilia sericata*; (2) the specific hypotheses (H) that we tested; and (3) the experimental methods that we applied to test these hypotheses.

H	Concise hypothesis statement	Method used
Do flashing cues from moving wings allow detection of potential mates?		
H1 (a)	Males discern between mates and males	Behavioural bioassay
H1 (b)	Wing movement is a mate recognition factor	Behavioural bioassay
H2 (a)	Wings are thin-film reflectors	TEM*
H2 (b)	Wing produce light flashes during movement	High speed video
H3	Wing flashing cues are absent or reduced under diffuse light	High speed video
Do optic effects produced by moving wings serve as mate recognition cues		
H4 (a)	Wings produce optic effects (Wing interference patterns, UV or polarized-light reflections)	Colour photography, polarimetry
H4 (b)	Optic effects of wings provide mate recognition cues	Behavioural bioassays with painted wings
Does the frequency of wing flashing light cues serve as mate recognition cues?		
H5	Frequency of wing flashes is gender-specific	Recording of wing beat sound

*Transmission Electron Microscopy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Stand
b
Camera
Lens
Reflector
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c
Camera
Lens
Ping pong ball
LED
LED
LED
LED

Figure 4.1. **Graphical illustrations depicting: (a) a T-bar (vertical stand: 3.5 cm tall; horizontal bar: 7.5 cm long) with two *Lucilia sericata* stimulus flies (SF) mounted on their abdominal ventrum to leave their legs without support and thus induce a wing fanning response; (b, c) the experimental design for high-speed video recordings of abdomen-mounted, wing fanning flies under direct light (b) and diffuse light (c). Flies were illuminated by one or four 100-watt cool LEDs. See methods for further details.**

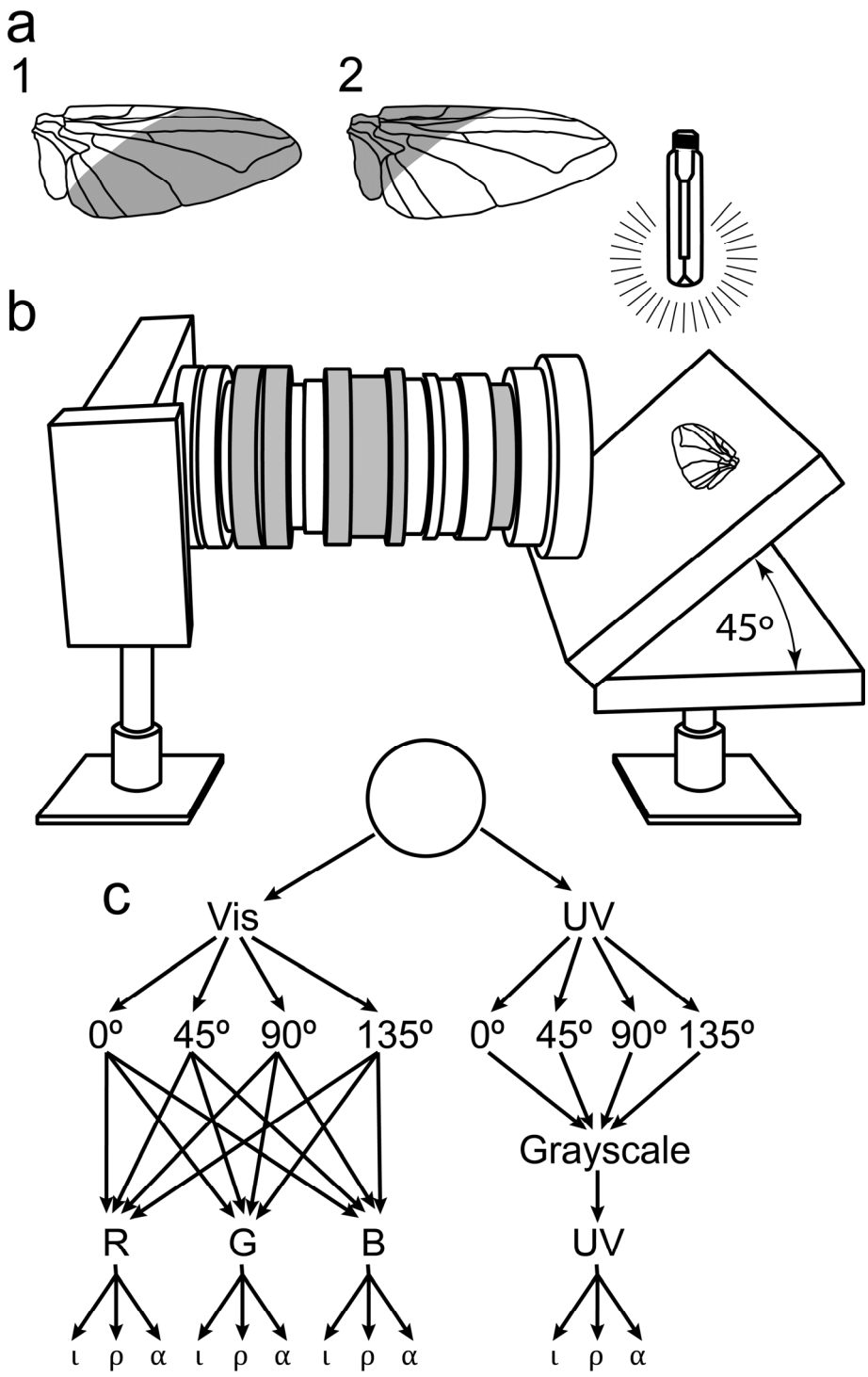
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Figure 4.2. **Graphical illustrations depicting: (a1) wings of a *Lucilia sericata* female spray-painted to (*i*) occlude potential Wing Interference Patterns (WIPs), UV- and polarized-light reflections and (*ii*) to test the resulting behavioural responses by males, and (a2) corresponding sham-sprayed wings; (b) the experimental design to study optic effects (WIPs, ultra-violet (UV) or polarized-light reflections) produced by wings, deploying a custom-designed camera and lens for enhanced UV sensitivity, and a metal halide lamp for illumination; (c) the flow chart for recording (*i*) (polarized) light reflections off wings in the human-visible (Vis) and UV ranges, (*ii*) four maximum axes of transmission from vertical (0°, 45°, 90°, and 135°), (*iii*) information in the red (R), blue (B), and green (G) colour channels, and (*iv*) the intensity (ι),degree (ρ) and angle (α) of polarization of *L. sericata* wings. See methods for further details.**

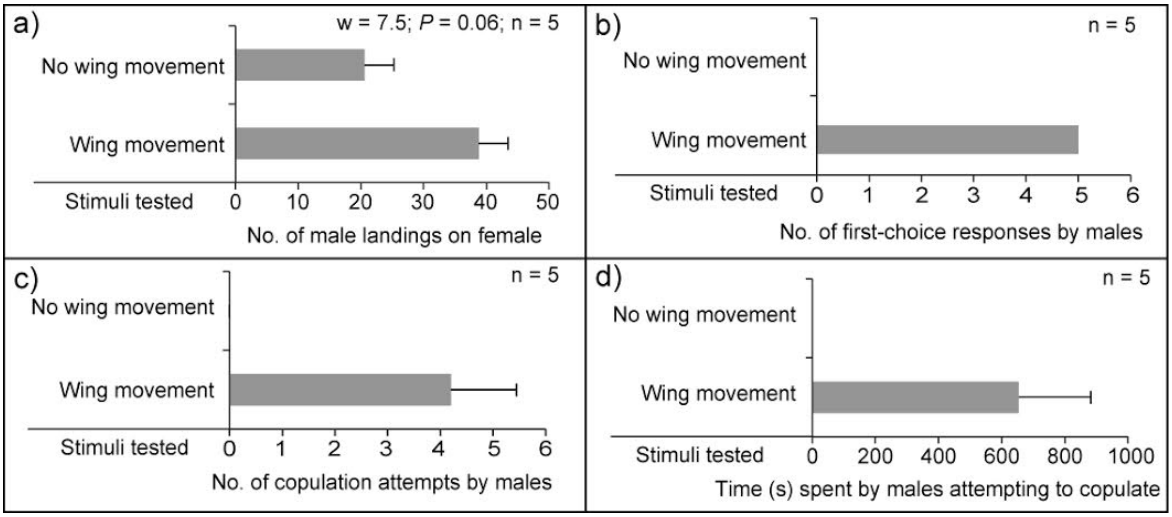

Figure 4.3. **(a-c) Mean cumulative number (+ SE) of landings by virgin *Lucilia sericata* males on a conspecific male or female (a), the number of their first-choice responses (b), and copulation attempts (c), and (d) the time (+ SE) males spent attempting to copulate with the female. In each 20-min replicate (n = 9), the male and the female were abdomen-mounted on a metal T-bar (Fig. 1 a; see methods for detail). In a, the female did not receive significantly more than 50% of the males' responses (two-tailed Wilcoxon signed-rank test, P = 0.0547). In b, c and d, females received 100% of the males' responses, and thus data were not analyzed statistically).**

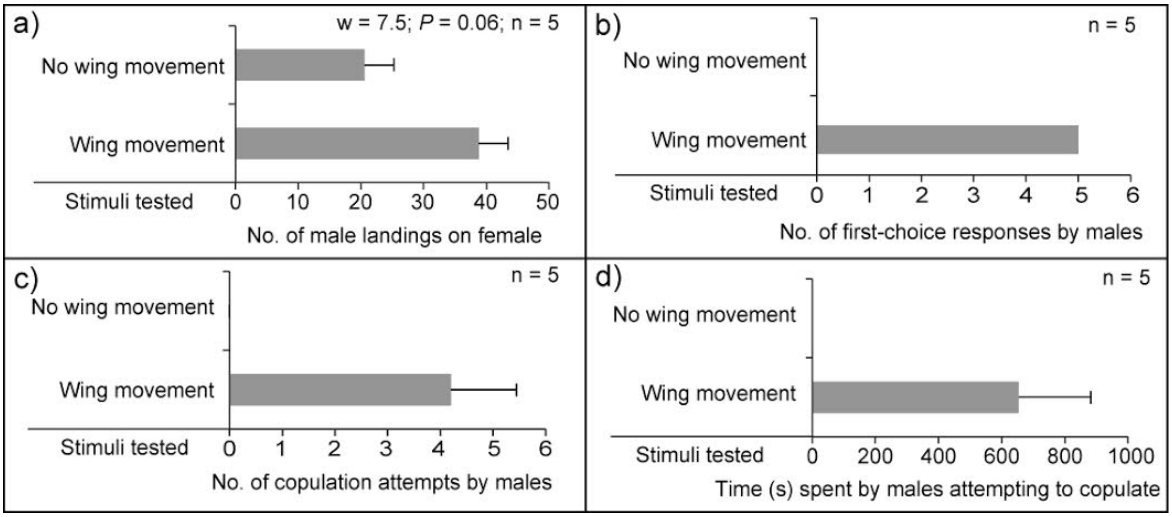

Figure 4.4. **(a-c) Mean cumulative number (+ SE) of landings by virgin *Lucilia sericata* males on a treatment or control female (a), the number of their first-choice responses (b), and copulation attempts (c), and (d) the time (+ SE) males spent attempting to copulate with a female. In each 40-min replicate (n = 5), the two females were abdomen-mounted on a metal T-bar (Fig. 1a), and the randomly assigned treatment female had immobilized wings (see methods for detail). In a, the female with functional wings did not receive significantly more than 50% of the males' responses (two-tailed Wilcoxon signed-rank test, P=0.06). In b, c and d, females with functional wings received 100% of the males' responses, and thus data were not analyzed statistically.**

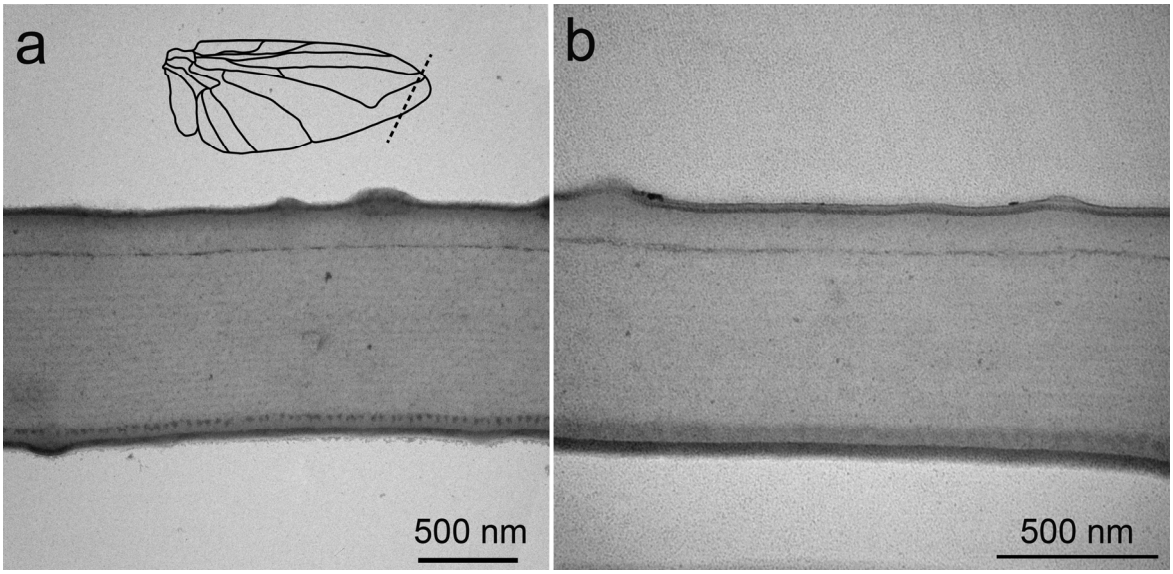

Figure 4.5. **Transmission electron micrographs (TEMs) depicting cross sections of wings of a *Lucilia sericata* female (a) and male (b). TEMs were taken near the wing tip as indicated by the dashed line on the wing insert in (a). The TEMs reveal that the dorsal and ventral membranes of the wing are fused forming a single thin layer.**

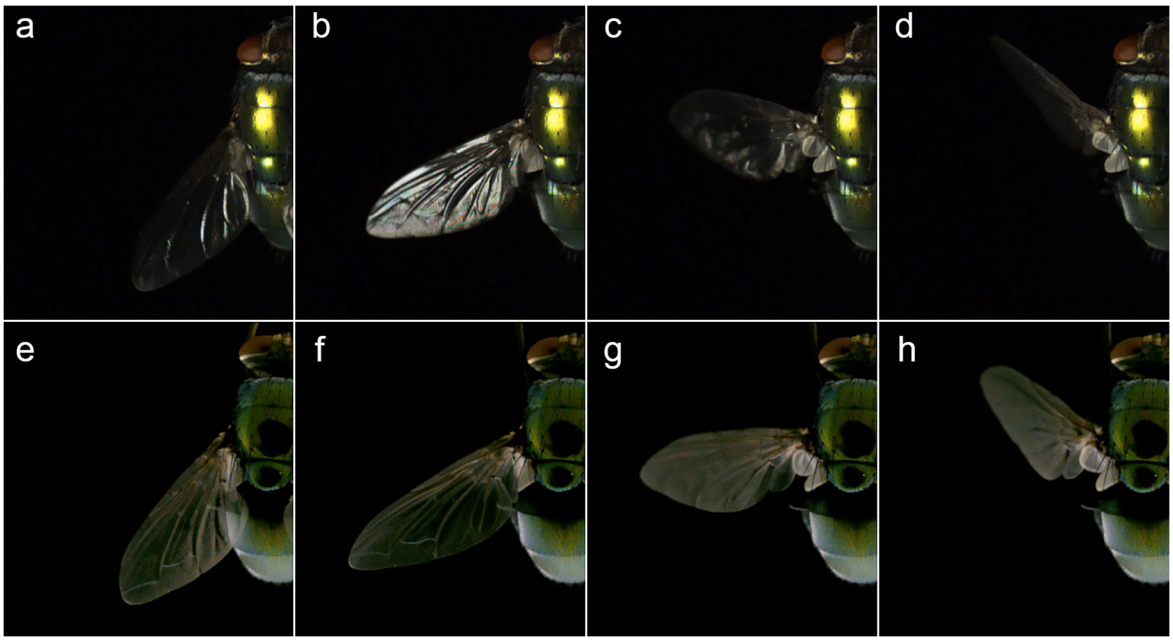

Figure 4.6. **Single-frame photographs of fanning wings of abdomen-mounted *Lucilia sericata* females (Fig. 1 a) taken from high speed video recordings (15,000 frames per second) under direct light (Fig. 1 b) or under diffuse light (Fig. 1 c). Photographs in the upper row (Fig. 6a-d) reveal changes in the intensity of light reflected off the wing as it rotates during wing fanning, thus causing a flashing light effect that may serve as a mate recognition cue. Photographs in the lower row (Fig. 6 e-h) fail to reveal any flashing light effect.**

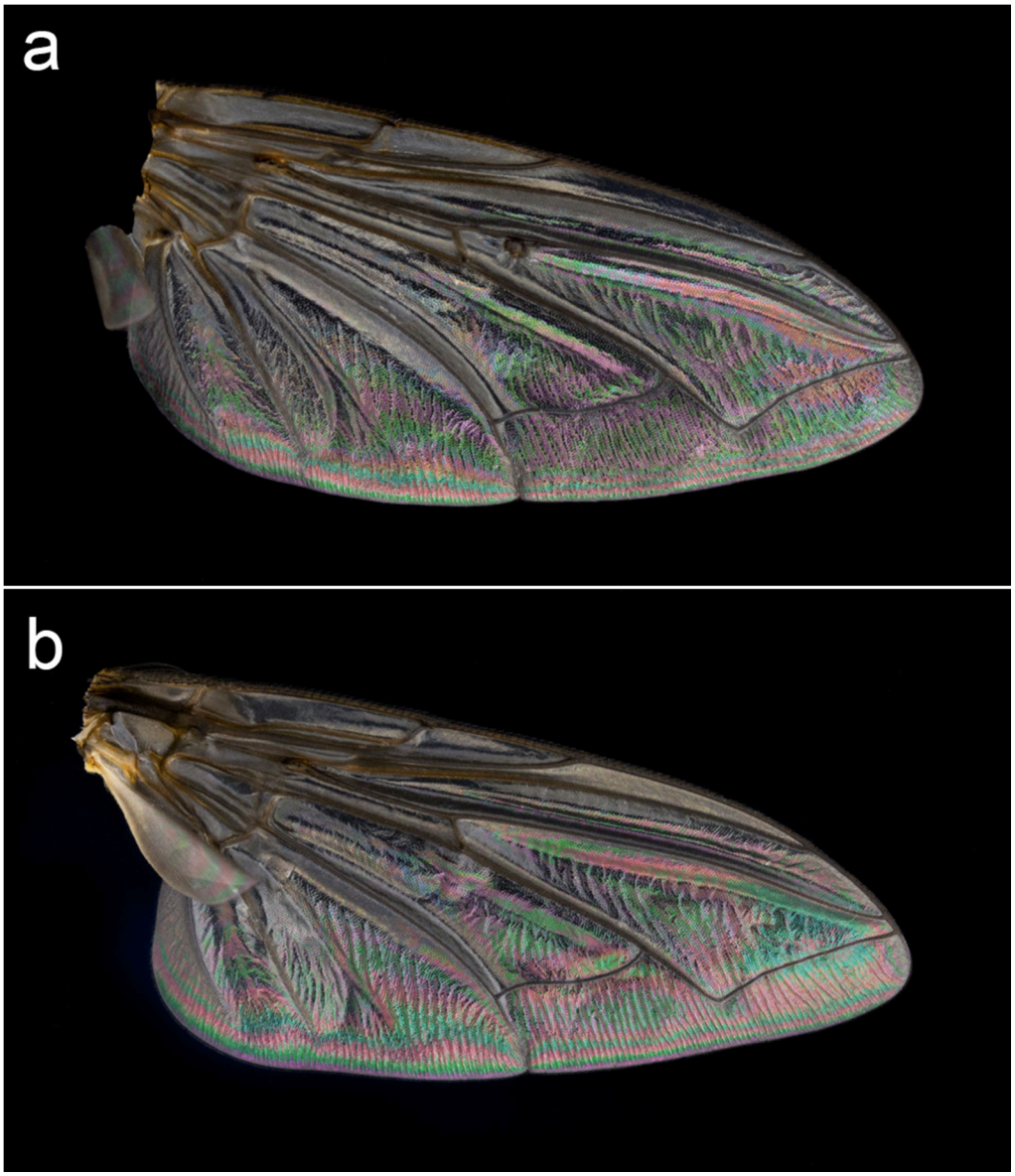

Figure 4.7. **Photographs of a female (a) and a male (b) *Lucila sericata* wing revealing wing interference patterns (see text for detail). Note the colour inversion at the wing tip, particularly the magenta spot on the female wing and the green spot on the male wing.**

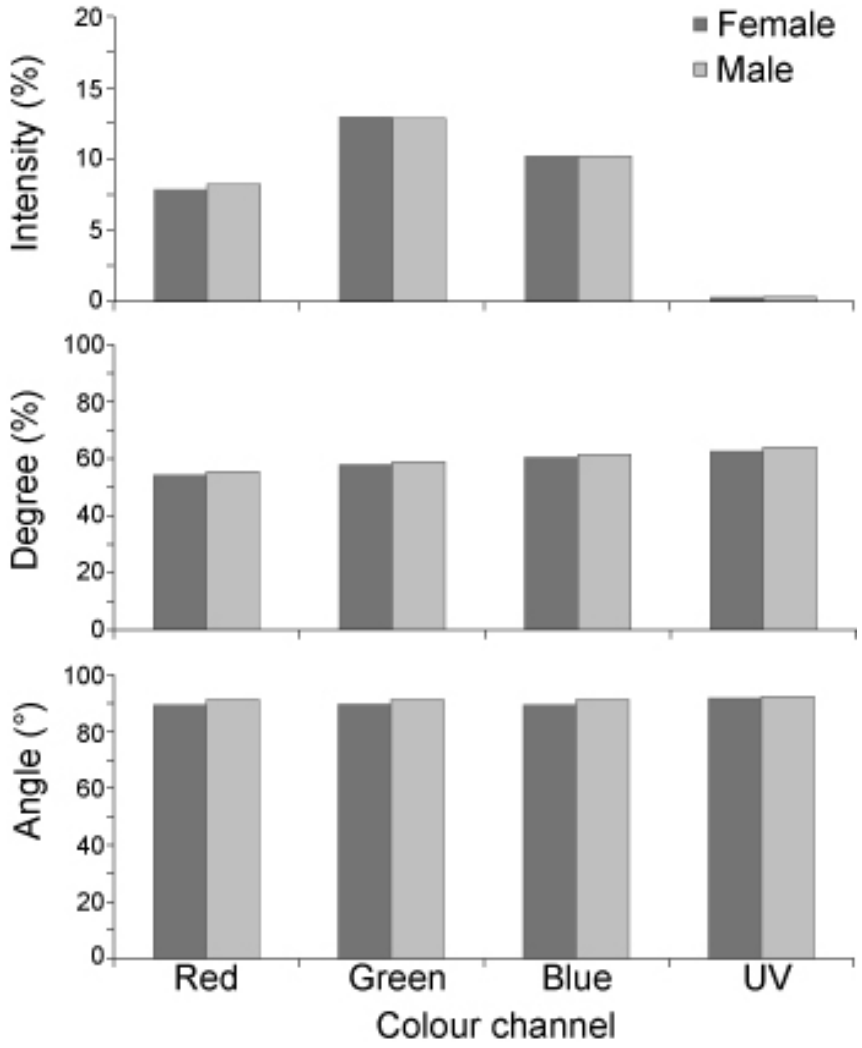

Figure 4.8. **Mean intensity (ı; top), degree (ρ; middle) and angle (α; bottom) of linearly polarized light reflected off the wings of *Lucilia sericata* males (light bars, n = 2) and females (dark bars, n = 2) in the red, green, blue and UV channel of electromagnetic wavelengths. Note: (1) Both male and female wings reflect light in the visible and UV range [intensity (ı): red ≈ 8%, green ≈ 13%, blue ≈10%, UV ≈ 0.35%]; (2) across all four channels the reflected light is about 60% polarized (ρ), at an angle (α) of 90 °; (3) Polarimetry images of male and female wings did not reveal any sexual dimorphisms in the intensity, degree, or angle of polarization; (4) Intensity measurements are relative to the number of photons that saturate the camera sensor (~7900 photons) and are not an absolute value of reflectance.**

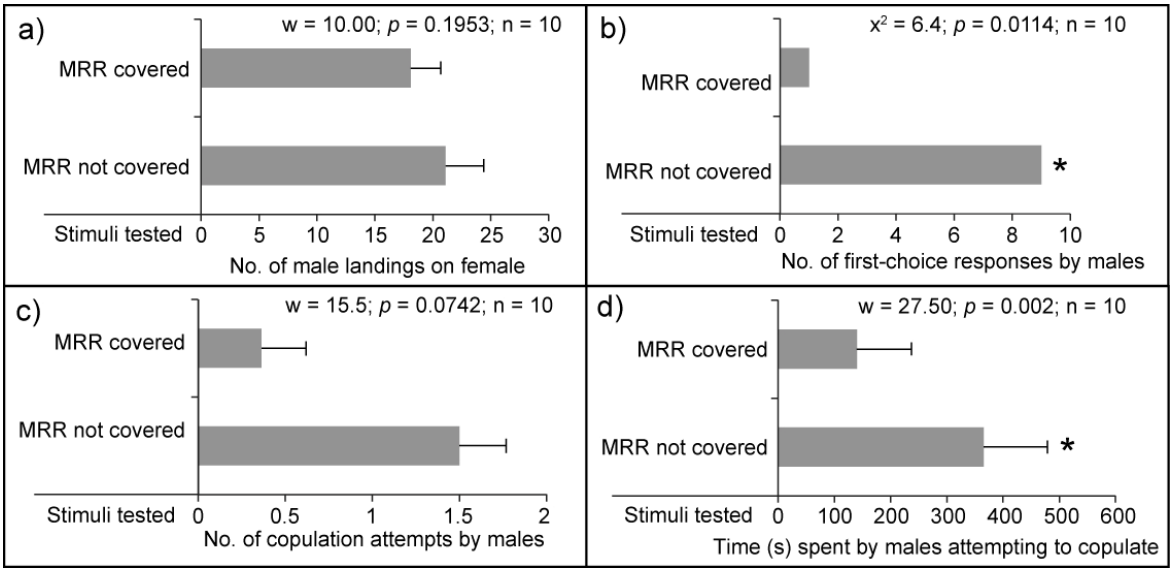

Figure 4.9. **(a-c) Mean cumulative number (+ SE) of landings by virgin *Lucilia sericata* males on a treatment or control female (a), the number of their first-choice responses (b), and copulation attempts (c), and (d) the time (+ SE) males spent attempting to copulate with a female. In each 20-min replicate (n = 10), the two females were abdomen-mounted on a metal T-bar (Fig. 1 a), and the randomly assigned treatment female had the most reflective region (MRR) of her wings (Fig. 2 a1), rather than the base of her wings (Fig. 2 a2), spray-painted (see methods for detail). In b and d, the asterisk indicates a significant preference by males for the control female (chi-squared test, $p = 0.0114$ for (b); two-tailed Wilcoxon signed-rank test, $p = 0.002$ for (d)).**

Chapter 5.

Bimodal cue complex signifies suitable oviposition sites to gravid females of the common green bottle fly

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5.1. Abstract

Gravid females of the common green bottle fly, *Lucilia sericata* Meigen (Diptera: Calliphoridae), readily locate recently deceased vertebrates as oviposition sites, particularly when these animals have been injured. We investigated semiochemical and visual cues that mediate attraction of gravid females to fresh rat carrion. Female flies were more strongly attracted to incised rat carrion than to intact carrion. They were also attracted to Porapak Q headspace volatile (HSV) extract of incised rat carrion. Analyzing aliquots of Porapak Q HSV extract by gas chromatographic-electroantennographic detection revealed nine components [phenol, *para*- and/or *meta*-cresol, guaiacol, dimethyl trisulfide (DMTS), phenylacetaldehyde, (*E*)-2-octenal, nonanal, and tetramethyl pyrazine] that consistently elicited responses from blow fly antennae. In laboratory experiments, a synthetic blend of these nine components was as attractive to gravid females as Porapak Q HSV extract, but blend attractiveness was due entirely to DMTS. In both laboratory and field experiments, increasing concentrations of DMTS attracted increasingly more flies. Coupled with DMTS, carrion-type colour cues (dark red, black) were more effective than bright colour cues (white, yellow) in attracting flies. In field experiments, dark traps baited with DMTS captured a total of 214 calliphorid flies (200 *L. sericata*, 10 *Lucilia illustris* Meigen, three *Calliphora vicina* Robineau-Desvoidy, one *Calliphora vomitoria* L.), all of

which were gravid females. These results support the conclusion that DMTS and dark colour represent a bimodal cue complex that signifies suitable oviposition sites to gravid calliphorid females, particularly *L.sericata.*

5.2. Introduction

Vertebrate carrion is a transient resource that undergoes temporary and progressive colonization by scatophagous and necrophagous insects (Byrd & Castner, 2010). The sequential colonization alters nutritional and physical characteristics of the resource (Payne, 1965; Carter et al., 2007; George et al., 2012; Richards et al., 2013), and causes progressive changes in its odourant composition (e.g., Takács et al., 2001; Forbes, 2008; Paczkowski et al., 2012; Johansen et al., 2014).

As an ephemeral resource, carrion is exploited by guilds of insects that respond to specific decomposition semiochemicals. Although the decomposition process is continuous, the physical and chemical changes can conceptually be assigned to distinct stages (Kreitlow, 2010) with characteristic odour profiles (Dekeirsschieter et al. 2009; Vass 2012; Vass et al., 2012) and specific guilds of responding insects (Payne, 1965; Anderson & Vanlaerhoven, 1996; Villet et al., 2010). In the first (fresh) and second (bloated) stage of carrion decomposition, blow flies (Calliphoridae), flesh flies (Sarcophagidae), and muscid flies (Muscidae) arrive, responding to nitrogen- or sulfur-containing compounds (Nilssen et al., 1996; Morris et al., 1998b; Aak et al., 2010; Frederickx et al., 2012; Paczkowski et al., 2012), small alcohols (Casana-Giner et al., 1999; Frederickx et al., 2012; Paczkowski et al., 2012; Johansen et al., 2014), and acids (Jones et al., 1976; Broce, 1980; Casana-Giner et al., 1999; Frederickx et al., 2012). During the third (active decay) stage, adult blow flies are absent and large maggot masses prevail. In the fourth (post-decay/dry) stage, checkered beetles (Cleridae), skin beetles (Dermestidae), and scarabid beetles (Scarabidae) take over. Clothes moths (Tineidae) attracted by saturated aldehydes (Takács et al., 2001) are other frequent inhabitants of the dry stage of the carrion. In the final (skeletal remains) stage, most insects of the above-mentioned taxa have disappeared and only mites remain.

Female blow flies lay their eggs not only on recently deceased animals, but also on injured live animals or exposed meat (Williams, 2009; Byrd & Castner, 2010). The association of calliphorid fly larvae with vertebrate wounds has been known since

antiquity, with the Old Testament being the oldest written report of a man whose wound was infested with fly larvae (Zumpt, 1965). The flies' response to wounds is mediated, in part, by bacterial odours emanating from wounds (Emmens & Murray, 1982; Chaudhury et al., 2002). This implies that wounded deceased animals develop bacterial odours more quickly than intact deceased animals, and thus are located quickly by gravid female flies seeking oviposition sites.

Blow flies exploit both semiochemical and visual cues when they seek carrion resources (Spivak et al., 1991; Torr & Hall, 1992; Wall & Fisher, 2001; Wertheim et al., 2005; Muller-Schwarze, 2006; Aak & Knudsen, 2011). Semiochemicals from bacterial decomposition of carrion induce activation, upwind orientation, and landing responses by flies (Ashworth & Wall, 1994; Chaudhury et al., 2010). Visual cues facilitate orientation of flies towards carrion resources at short range (Smith et al., 2002). Blow flies differentiate among colours (Wall & Smith, 1996; Briscoe & Chittka, 2001), but their colour preference remains inconclusive. Yellow-coloured traps were more attractive than blue, pink, or green traps to naive *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae) (Lee, 1937). Similar results were obtained in other studies with *L. cuprina* and *Lucilia sericata* Meigen (Diptera: Calliphoridae) (Fukushi, 1989; Wall et al., 1992; Wall & Smith, 1996). In experiments with different trap colours, females of *L. sericata* and *Wohlfahrtia magnifica* (Schiner) were mainly attracted to dark colours (Hall et al., 1995), whereas other species of blow flies including *L. cuprina* and *Lucilia* spp. did not reveal any colour preference (Mello et al., 2009). These inconsistent results can be attributed to the different physiology--driven decisions based on reproductive status or ontogenetic development of responding flies, and different types of baits (swormlure-4, liver, sardine, un-baited), traps (light, tanglefoot, inverted funnel), or physical properties of colours (hue, intensity, brightness) that were tested in these studies.

Interactive effects of visual and olfactory cues on foraging blow flies (Spivak et al., 1991; Torr & Hall, 1992; Wall & Fisher, 2001; Aak & Knudsen, 2011) are likely also context-, gender-, and state-specific. Nectar-foraging males and females would be expected to respond to a combination of floral colours and semiochemicals, whereas mated gravid females seeking oviposition sites would be expected to respond to carrion

odour and dark colours (gray, red, brown, black) that are typically associated with animal fur.

Many semiochemicals associated with fresh animal carrion have been identified and tested individually for their ability to elicit electrophysiological and/or behavioural responses from blow flies (Nilssen et al., 1996; Park & Cork, 1999; Aak et al., 2010; Frederickx et al., 2012; Paczkowski et al., 2012). Yet, entire headspace volatile blends of fresh carrion, or all antennally active components in such blends, have not yet been tested to determine the key semiochemical(s) that attract gravid female blow flies.

In this study, we worked with *L. sericata*, an early responder to animal corpses (Davis, 1928; Cragg, 1956; Hall & Doisy, 1993; Cragg & Hobart, 1995; Byrd & Castner, 2010) and common representative blow fly species in the northern hemisphere (Hall, 1948; Hall & Townsend, 1977). Our specific objectives were to: (1) bioassay attraction of flies to intact and incised carrion; (2) obtain headspace volatile (HSV) extract of attractive carrion and bioassay its attractiveness to flies; (3) identify candidate semiochemicals in bioactive HSV extracts; (4) determine the essential component(s) in a synthetic blend of candidate HSV semiochemicals; and (5) investigate potential interaction between the key semiochemical(s) and colour cues for attraction of flies.

5.3. Materials and methods

5.3.1. Source of carrion and experimental flies, and general bioassay procedure

Norway rats, *Rattus norvegicus* (Berkenhout), were selected as the carrion source due to their availability and relatively small body size. Live rats were purchased by, and housed in, the Animal Care Facility of Simon Faser Universiy (SFU) (Permit # 1042-12). To bioassay the response of flies to recently-deceased animals, and to capture the volatiles of such animals, rats were CO_2-euthanized. To simulate the death of an injured rat, the body cavity of some rats was opened by incision immediately post mortem by CO_2-euthanizaion. The 2.5-cm-wide and 5-cm-long T-shaped incision severed the skin

below the ribs and from the sternum toward the pelvis, exposing but not puncturing the intestines and not causing visible bleeding.

Lucilia sericata was reared in SFU's insectary, starting a new colony with wild-caught flies every 12 months. Flies were kept under a L16:D8 photoperiod, at 30-40% r.h., and 23-25 °C. They were provided with water, milk powder, and sugar *ad libitum*, and given access to bovine liver (Supreme Meat Supplies, Burnaby, BC, Canada) for 15 min daily. Adult flies were aged ≤ 36 h or 7-13 days post eclosion before they were tested in bioassays.

For each experimental replicate, 100 cold-sedated flies (Table 1) were placed into an aluminium wire mesh cage (61 × 61 × 61 cm) with a plated grey base (BioQuip Products, Compton, CA, USA)(Figure 1A). Each cage was illuminated from above with fluorescent lights (F32TA; Phillips, Amsterdam, The Netherlands). All flies were given 2 h to acclimate prior to inserting test stimuli and were not retested in another experiment. Considering that the response propensity of flies can vary among days and thus make experimental treatment effects less apparent, the same number of replicates of each of experiments 4-19, and of experiment 20, were run concurrently (in parallel) on any given day.

Objective 1: Bioassay attraction of flies to intact and incised carrion

Laboratory experiment 1 (n = 10; Table 1) tested three stimuli: (1) an intact rat 1-4 h post mortem, (2) a rat 1-4 h post mortem incised immediately after CO_2-euthanization, and (3) a blank control. Each stimulus was randomly assigned to one of three white paper Solo cups (0.5 l; 9 × 8.5 cm) (Solo Cup Operating Corporation, Lake Forest, IL, USA), which were covered with cheesecloth (VWR, Radnor, PA, USA) to standardize visual cues, and placed equidistant (30 cm) to each other in triangular configuration into the cage (Figure 1A). Each cage contained 100 13-day-old gravid flies. The number of flies present on the cheesecloth of each cup was counted every 5 min for 3 h and averaged for each replicate.

Objective 2: Obtain headspace volatile (HSV) extract of attractive carrion and bioassay its attractiveness to flies

Separate groups of 5-6 intact or cut rat carrion (see above) were placed in a Pyrex® glass chamber (34 cm high × 12.5 cm wide). A water aspirator drew charcoal-filtered air at 0.5 l min^{-1} for 8 h through the chamber and through a glass column (6 mm outer diameter × 150 mm) containing 200 mg of Porapak-Q™ (50-80 mesh) adsorbent (Byrne et al., 1975). Rat-derived volatiles captured on Porapak were desorbed with 2 ml of pentane. Aliquots of Porapak HSV extract were bioassayed for the response of flies (see below), and bioactive extracts were analyzed (see below).

Experiment 2 tested HSV extract of rat carrion at 1 rat-hour-equivalent (the amount of volatiles given off one incised rat carrion during 1 h). The design of experiment 2 (n = 10; Table 1) was similar as described for experiment 1 (objective 1) with the exception that (i) only two (instead of three) stimuli were tested, (ii) Porapak Q HSV extract of incised rat carrion (see above) served as the treatment stimulus, (iii) pentane was the control stimulus, (iv) aliquots of test stimuli were pipetted onto filter paper (Whatman™, Maidstone, UK) which was placed at the bottom of Solo cups, and (v) the number of fly landings on treatment or control Solo cups within 5 min was recorded as response criterion. This brief bioassay period took into account that components in headspace volatile extracts may disseminate quickly and thus challenge the flies' ability to discern between test stimuli for > 5 min periods.

Objective 3: Identify candidate semiochemicals in attractive HSV extract

Aliquots of Porapak-Q HSV extract of incised rat carrion were analysed by gas chromatographic-electroantennographic detection (GC-EAD) and GC-mass spectrometry (MS), with procedures and equipment previously described in detail (Arn et al., 1975; Gries et al., 2002). For GC-EAD recordings (n = 10), an antenna was carefully pulled from the head of a 7–13-day-old female fly and suspended between two glass capillary electrodes (1.0 × 0.58 × 100 mm) (A-M Systems, Carlsborg, WA, USA) filled with saline solution (Staddon & Everton, 1980). Volatile components that elicited responses from at least seven out of 10 different antennae were considered candidate semiochemicals. The Hewlett Packard 5890 gas chromatograph (GC) was fitted with a DB-5 GC column [30 m × 0.32 mm inner diameter (i.d.); J&W Scientific, Folsom, CA, USA)]. Helium was used as

the carrier gas (35 cm s^{-1}) with the following temperature program: 50 °C for 5 min, 20° min^{-1} to 280 °C. The injector port and flame ionization detector (FID) were set at 250 °C. Candidate semiochemicals were analyzed by a Saturn 2000 Ion Trap GC-MS operated in full-scan electron impact mode and fitted with a DB-5 GC-MS column (50 m × 0.25 mm i.d.). Helium was used as the carrier gas (35 cm s^{-1}) with the following temperature program: 50 °C for 1 min, 10 °C min^{-1} until 280 °C (10 min). The injector port and ion trap were set at 250 °C and 260 °C, respectively. Components that elicit responses from fly antennae were identified by comparing their retention indices (Van den Dool & Kratz, 1963) and mass spectra with those reported in the literature [components 1-6 (Figure 4) (Adams, 1989), components 7-9 (Figure 4) (Jennings, 1980)] and with those of authentic standards.

To confirm the structural assignment of candidate semiochemicals and to prepare synthetic blends (see objective 4), the following compounds were purchased: phenol (>95% chemical purity), DMTS (>95%), phenylacetaldehyde (90%), *meta*- and *para*-cresol (99%), tetramethyl pyrazine (98%), and nonanal (95%) (Sigma Aldrich, St. Louis, MO, USA), guaiacol (98%) (Fluka™, Buch, Switzerland), and (*E*)-2-octenal (Bedoukian, Danbury, CT, USA).

Objective 4: Determine the essential component(s) in a synthetic blend of candidate HSV semiochemicals

The design of experiments 3-19 (n = 10 each) was as described for experiment 2 except that synthetic candidate carrion semiochemicals in pentane served as the treatment stimulus. Experiment 3 tested a synthetic blend (SB) of all components in HSV extracts of incised rat carrion that had elicited responses from fly antennae in GC-EAD recordings (see Results). SB components were prepared at ratios equivalent to those found in GC-MS analyses of HSV extracts and were tested at 10 rat-hour equivalents (see Table 1). SB included four alcohols [phenol, *para*- and *meta*-cresol (could not be separated), and guaiacol], three aldehydes (phenylacetaldehyde, (*E*)-2-octenal, and nonanal), dimethyl trisulfide (DMTS), and tetramethyl pyrazine (pyrazine). Added to this blend were several acids (isobutyric, 2-methylbutyric, butyric, amyl, isovaleric, and hexanoic) which were present in headspace volatile extracts but chromatographed poorly

and thus did not elicit consistent antennal responses in GC-EAD analyses. Furthermore, acids were previously found to be attractive to *L. sericata* (Frederickx et al., 2012).

With evidence that headspace volatile extract of incised rat carrion and SB were both effective in attracting flies (see Results), the next set of experiments was designed to determine the essential semiochemical(s) mediating the response of flies to SB. Predicting that the flies' response may be affected by their sex and developmental stage, 13-day-old gravid females, protein-deprived 3-day-old females, and protein-deprived 3-day-old males were tested separately in experiments 4-9, 10-14, and 15-19, respectively (Table 1). Parallel-run experiments 4-9 tested SB (experiment 4), and SB that lacked groups of organic chemicals, such as acids (experiment 5), alcohols (experiment 6), aldehydes (experiments 7), DMTS (experiment 8), and pyrazine (experiment 9). Each set of parallel-run experiments 10-14 and 15-19 was identical in design.

Objective 5: Investigate potential interaction between the key semiochemical(s) and colour cues for attraction of flies

With evidence that DMTS is a key semiochemical cue that attracts gravid female flies to rat carrion (see Results), and predicting that a dark colour cue is representative of most mammalian carrion sought by gravid flies, laboratory experiment 20 (n = 10) tested the effects of the amount of DMTS and associated colour cues on the response of gravid flies. DMTS was applied to filter paper placed at the bottom of Solo cups (see objective 2). The two treatments in each pairwise comparison tested DMTS at identical amounts (0, 1, 10, 100, or 1000 ng) but in combination with either a white or dark-red cheesecloth covering the Solo cup (see spectrometric profiles in Results) and thus contrasting by different degrees against the grey aluminum base plate of the cage. The number of fly landings on each of the two Solo cups within 5 min in each pairwise comparison was recorded as response criterion. Cheesecloth was dyed with dye (RIT, Stamford, CT, USA), using cocoa brown #20 (16 g, powder) and wine #10 (15 ml, liquid).

Three field experiments (experiments 21-23) were run on a dairy farm in Delta, BC, Canada (49°06'49"N, 123°02'05"W) along a fence line with green pasture on both sides of the fence. Each experiment had a randomized complete block design, and incorporated Oakstem traps (Figure 1B; Contech Enterprises, Delta, BC, Canada), which

were suspended from a fence so that the top of the trap was about 75 cm above ground. Traps were placed at 10-m intervals within and between blocks (replicates), and were baited with a 4-ml glass vial containing 900 µl of gelled paraffin oil with various concentrations of DMTS (see below). Gelled oil was prepared by stirring it with 1% (wt/vol) 12-hydroxystearic acid at 55 °C. The bottom of each trap was filled with soapy water to retain responding flies. Experiments were terminated by scoring for each species of fly the number, sex, and physiological status of specimens captured in each trap. To determine the physiological status of captured female flies, their oocytes were excised, viewed under microscope, and the stage of follicle development (Adams & Reinecke, 1979) was scored.

Experiment 21 (1-8 September 2012; n = 10) tested the effect of the amount of DMTS on attraction of flies. DMTS was admixed at 0, 0.1, 1, or 10% (wt/vol) to gelled paraffin oil (see above). All traps were covered with brown construction paper, with a strip of black electrical tape placed above and below the two openings of each trap to possibly enhance trap attractiveness (Diclaro et al., 2012). Experiments 22 and 23 tested the effect of trap colour on attraction of flies, with a 10-% DMTS lure in each trap (see above). In experiment 22 (18-21 September 2012; n = 12), the two traps in each replicate were covered with either black or yellow construction paper, and in experiment 23 (23-28 September 2012; n = 12), they were covered with black or white construction paper.

5.3.2. Spectrometric profiles and thermographic images of test stimuli

The spectrometric profiles of red or white cheesecloth in experiment 20, and of brown, black, white, or yellow construction paper covering traps in experiments 21-23, were recorded with a spectrometer (Ocean Optics, Dunedin, FL, USA). Because sun-exposed construction paper faded slightly over time, spectrometric profiles were recorded at the onset and end of experiments 22 and 23, which were designed to test the effect of colour on the flies' response. Moreover, predicting that trap colour may affect trap temperature which – in turn – may affect release rates of DMTS and the flies' response, thermographic images of traps with white, yellow, or faded yellow, or black or faded black construction paper (n = 5 each) were recorded, using a FLIR SC620 Thermal Imaging

Camera (Flir, Wilsonville, OR, USA). Prior to recordings, traps were exposed at mid-day to sunlight for 30 min at an air temperature of 24.9-25.6 °C.

5.3.3. Statistical analysis

In experiments 1-19, we analyzed the mean proportion of fly landings on test stimuli by one-tailed t-test, expecting a preferred stimulus to induce at least >50% of the flies' responses in three-choice experiment 1, and to induce > 70% of the fly responses in two-choice experiments 2-19. These values were set high deliberately for conservative assessment of stimuli attractiveness.

In experiments 3-9 (gravid females), 10-14 (protein-deprived females), and 15-19 (protein-deprived males) (objective 4), we also subjected the absolute number of fly landings on the treatment stimulus to ANOVA followed by pairwise comparisons with Tukey's honest significant difference (HSD) test to determine significant differences in the flies' response to the complete blend, or partial blends, of synthetic candidate semiochemicals.

In experiment 20, we analyzed the response of flies to various concentrations of DMTS by one-way ANOVA, with the total number of fly landings on white and red cheesecloth as the response variable. We then performed pairwise comparisons using Tukey`s HSD test to determine significant differences in the flies' response to different DMTS concentrations. We also tested for an interactive effect between the concentration of DMTS and cheesecloth colour on the flies' response by one-way ANOVA. As the numbers of fly landings on each of the two test stimuli within a given replicate (cage) were not independent, we considered the differential between the number of landings on cups with red and white cheesecloth as the response variable.

In experiment 21, we analyzed trap captures of flies in response to the amount of DMTS by ANOVA followed by Tukey's HSD test. In Experiments 22 and 23, we analyzed trap captures of flies in response to colour by one-way ANOVA. We analyzed all data using JMP 10® (SAS Institute) for Windows® (Windows Corporation, Redmond, WA, USA).

5.4. Results

Objective 1: Bioassay attraction of flies to intact and incised carrion

In three-choice experiment 1, incised rat carrion received on average more fly landings (mean ± SE, 31.27 ± 1.09) than did intact rat carrion (8.35 ± 0.51) or the control stimulus (8.86 ± 0.6). Only incised rat carrion received significantly more than 50% of the flies' landing responses ($t = 4.05$, d.f. = 36, $P<0.0001$; Figure 2).

Objective 2: Obtain HSV extract of attractive carrion and bioassay its attractiveness to flies

In two-choice experiment 2, aliquots of Porapak Q HSV extract of incised rat carrion received 63.7 ± 5.94 fly landings (>70% of the flies' landing responses; $t = 5.18$, d.f. = 9, $P<0.001$) whereas the solvent control received only 9.5 ± 1.27 (Figure 3, Exp. 2), indicating that the essential semiochemical(s) associated with incised rat carrion were present in the extract.

Objective 3: Identify candidate semiochemicals in bioactive HSV extract

In GC-EAD analyses of HSV extract of incised rat carrion, nine components [phenol, *para*-and/or *meta*-cresol, guaiacol, dimethyl trisulfide (DMTS), phenylacetaldehyde, (*E*)-2-octenal, nonanal, and tetramethyl pyrazine] consistently elicited responses from blow fly antennae (Figure 4). Of these nine components, DMTS elicited the strongest EAD response.

Objective 4: Determine the essential component(s) in a synthetic blend of candidate semiochemicals from incised rat carrion

The synthetic blend (SB) of candidate semiochemicals (Table 1) from incised rat carrion received 96.6 ± 10.36 fly landings (>70% of the flies' landing responses; $t = 3.96$, d.f. = 9, $P<0.005$) whereas the solvent control received 19.8 ± 2.94 fly landings (Figure 3, Exp. 3). This SB was as effective as HSV extract of incised rat carrion in eliciting the flies' response ($U = 70$, d.f. = 1, $P = 0.131$; Figure 3), indicating that SB contained the key

semiochemical(s).

The response of gravid female flies to the complete SB, or to SBs lacking specific components, differed significantly ($F_{5,54}$ = 21.79, P<0.0001; Figure 5). The SB lacking aldehydes received significantly more landing responses by gravid female flies than did the complete SB (Figure 5, Exp. 7). In contrast, the SB lacking both dimethyl trisulfide (DMTS) and tetramethyl pyrazine, or the SB lacking just DMTS, received significantly fewer landing responses by gravid female flies than did the complete SB (Figure 5, Exps. 4, 8, 9). These results indicate that the activity of SB was due to DMTS and that the presence of aldehydes reduced the blend's attractiveness.

In contrast, the response of protein-deprived females (Exps. 10-14), or protein-deprived males (Exp. 15-19), to the complete SB, or to SBs lacking specific components, did not differ significantly (females: $F_{4,45}$ = 2.29, P>0.0742; males: $F_{4,45}$ = 1.634, P>0.1822; Figure 5), indicating that DMTS does not have the same signal function to protein-deprived females or males as it does to gravid female flies.

Objective 5: Investigate potential interaction between the key semiochemical(s) and colour cues for attraction of flies to carrion

In laboratory experiment 20, the concentration of DMTS had a significant effect on the landing response of flies ($F_{4,45}$ = 64.55, P<0.0001; Figure 6). DMTS at concentrations of 100- and 1000-ng was equally effective, and significantly more effective than at a 1- and 10-ng concentration, which statistically did not induce more landing responses than did the control stimulus.

There was significant interaction between the concentration of DMTS and cheesecloth colour ($F_{4,45}$ = 12.62, P<0.0001; Figure 6). Overall, flies landed more often on stimuli with red than with white cheesecloth but the extent of this preferential response varied with concentration, with the strongest preferential response occurring at 100 and 1000 ng of DMTS (Figure 6).

In (field) experiment 21, the concentration of DMTS had a significant effect on trap captures of flies ($F_{4,45}$ = 64.55, P<0.0001; Figure 7). The 10-% DMTS lure attracted 5.08 ± 1.54 flies, seven times more flies than the 1.0-% DMTS lure (0.75 ± 0.49) and 33 times more flies than the 0.1-% DMTS lure (0.17 ± 0.17).

In experiment 22, black traps baited with a 10% DMTS lure captured significantly more

blow flies (4.58 ± 2.0) than yellow traps (0.15 ± 0.083) baited with same lure (ANOVA: U = 20, d.f. = 1, P = 0.001; Figure 7). All captured blow flies were gravid females with fully developed eggs. Of the 55 blow flies captured in black traps, 53 were *L. sericata* and two were *Lucilia illustris* Meigen (Diptera: Calliphoridae). The yellow trap captured one *L. sericata* (Table 2). In experiment 23, black traps baited with a 10% DMTS lure captured significantly more blow flies (6.83 ± 2.91) than white traps (1.17 ± 0.64) baited with same lure (ANOVA: U = 41, d.f. = 1, P = 0.05). Of the 82 blow flies captured in black traps, 74 were *L. sericata*, five were *L. illustris*, two were *Calliphora vicina* Robineau-Desvoidy (Diptera: Calliphoridae), and one was a *Calliphora vomitoria* L. (Diptera: Calliphoridae). Of the 14 blow flies captured in white traps, 13 were *L. sericata* and one was *L. illustris* (Table 1).

5.4.1. Spectral reflectance and thermography of test stimuli

Spectral reflectance from test stimuli is reported in Figures 6B and 7B. It is noteworthy that field-tested yellow and black faded slightly over time but that the reflectance profiles of original and faded colour cues were similar. In thermographs, the mean temperature of traps varied with the colour of the construction paper covering a trap, as follows: black: 25.6 °C; faded black: 24.7 °C; yellow: 22.6 °C; faded yellow: 22.9 °C; and white: 22.0 °C.

5.5. Discussion

Our data support the conclusion that (1) in a choice setting, incised rat carrion is more attractive to blow flies than intact rat carrion, (2) headspace volatiles of incised rat carrion attract blow flies, (3) dimethyl trisulfide (DMTS) is a key semiochemical in headspace volatile extract of incised rat carrion that mediates attraction of gravid flies, and (4) DMTS is more effective in attracting flies when combined with dark colour cues (red, black) rather than bright colour cues (white, yellow).

The profound preference of blow flies for incised over intact rat carrion (Figure 2) is linked to the difference in odour intensity. Incised carrion released significantly larger quantities of odourants, including six times more DMTS. The experimental incision of rat

carrion mimicked a wound or physical injury of an animal eventually causing death. The wound surface apparently facilitates colonization of specific bacteria that break down nutrients and in the process release volatiles that attract blow flies (Morris et al., 1998a; Chaudhury et al., 2002, 2010; Schulz & Dickschat, 2007; Tomberlin et al., 2012). However, the type of volatiles these bacteria produce, and the manner in which flies respond, appear bacterium- or strain-specific. For example, of various bacterial strains isolated from the healthy breech mucosa and myiatic wounds of ewes, two bacterial isolates [*Rhodococcus fascians* Tilford (Dowson) and *Mycobacterium aurum* (Tsukamura)] from the brim of wounds attracted significantly more male and female flies of *W. magnifica*, *L. cuprina*, *L. sericata,* and *Musca domestica* L. (Diptera: Muscidae) than any other selected bacterial strains (Khoga et al., 2002). Interestingly, both *R. fascians* and *M. aurum*, but no other selected strains, produced DMTS (Khoga et al., 2002). This makes it very likely that DMTS in headspace volatiles of incised rat carrion (Figure 2) is also produced by bacteria (see Paczkowski & Schuetz, 2011).

To determine the key semiochemical(s) in incised rat carrion that attract blow flies, we took a very systematic and proven effective approach. We first ascertained that not only incised rat carrion but also Porapak Q headspace volatile (HSV) extract thereof attracted flies. This was important because some of the reported odourants emanating from animal carrion, such as methanthiol or dimethyl sulfide, are rather volatile and may not readily absorb on Porapak Q or other types of absorbents. With strong attraction of flies to Porapak Q HSV extract (Figure 4), we then screened aliquots of the extract by GC-EAD on the premise that only those components that elicit an antennal response can possibly induce a chemotactic behavioural response of flies, and thus ought to be included in a synthetic blend for bioassays. GC-EAD analysis of Porapak Q HSV extract is also advantageous in that it draws attention to all candidate semiochemicals in the extract irrespective of their relative abundance. For example, although DMTS was present in only trace quantity, we considered it a prime candidate semiochemical based on its strong antennal (EAD) activity (Figure 4). With comparable attraction of flies to HSV extract of incised rat carrion and to a synthetic blend of all EAD-active candidate semiochemicals (Figure 3), we proceeded to determine the essential semiochemical(s) in the synthetic blend. We tested the synthetic blend at a 10-fold higher amount than the blend of natural carrion (10 instead of 1 rat-hour-equivalents), considering that natural

carrion keeps emitting semiochemicals over time whereas synthetic blends quickly evaporate from filter paper. Taking the most efficient approach to determine the essential semiochemical(s) in the synthetic blend (Byers 1992), we compiled partial blends of candidate semiochemicals that lacked specific groups of organic compounds such as alcohols and aldehydes, and then compared their relative attractiveness to the complete blend (Figure 5). This approach revealed quickly and clearly that blends lacking DMTS were as unattractive to gravid females as unbaited controls, indicating that DMTS is a key semiochemical of (incised) fresh rat carrion. However, we do not discount potentially additive effects of other carrion semiochemicals. For example, even though synthetic blends without DMTS were as ineffective as control stimuli based on conservative statistical analyses (see experiments 8 and 9 in Figure 5), these partial synthetic blends still received more fly landings than control stimuli, likely due to other (albeit less important) semiochemicals in the blend.

Testing partial blends also revealed that the presence of aldehydes interfered with optimal blend attractiveness. This is not surprising because aldehydes seem to signal the dry stage of carrion decomposition, which is no longer suitable for oviposition by blow flies. Indeed, the aldehyde nonanal and geranylacetone are key dry-animal-pelt semiochemicals that attract both the webbing clothes moth, *Tineola bisselliella* (Hummel) (Takács al., 2001), and its larval parasitoid *Apanteles carpatus* (Say) (Takács al., 1997).

That DMTS signals the presence of suitable oviposition sites was supported by laboratory and field data showing that DMTS attracted only gravid, oviposition-site seeking females, but not protein-deprived females or males (Figure 5, Table 2). Every single specimen of *L. sericata*, *L. illustris*, *C. vomitoria*, and *C. vicina* captured in DMTS-baited traps in field experiment 21-23 was a gravid female fly with fully developed eggs (Table 1). In related studies, DMTS was tested as part of a semiochemical blend or stimulus complex that attracted calliphorid flies (Nilssen 1996; Aak et al., 2010, 2011, 2012; Frederickx et al., 2012; Paczkowski et al., 2012; Johansen et al., 2014).

Although both blow flies and flesh flies are the first and most dominant species of flies to arrive at fresh animal carrion (Payne, 1965; Smith, 1986; Hall & Doisy 1993; Anderson & VanLaerhoven, 1996; Tabor et al., 2005; Byrd & Castner, 2010), DMTS in

field experiments 21-23 attracted primarily blow flies (Table 1). It is possible that flesh and muscid flies were simply absent from the experimental site, or that they preferentially responded to other oviposition sites such as animal faeces and rotting vegetation. Alternatively, flesh and muscid flies may respond to a different carrion cue, or to multiple- instead of single-component blends (Cossé & Baker 1996). For example, a blend of ethyl acetate, acetic acid, and ethanol attracted the flesh fly *Sarcophaga carmia* Meigen (Casãna-Giner et al., 1999), although none of these three chemicals was a key constituent of carrion in our study. Similarly, a blend of 3-methylindole, butanoic acid, and DMTS - but neither of these compounds alone – induced landing of house flies, *Musca domestica* L., on a source (Cossé & Baker 1996). That any muscid flies were absent in captures of DMTS-baited traps (Table 1) is somewhat surprising because DMTS is known to elicit electrophysiological and behavioural responses from various muscid flies, including the stable fly, *Stomoxys calcitrans* L. (Jeanbourquin & Guerin, 2007; Tangtrakulwanich et al., 2011) and *Hydrotaea anxia* Zetterstedt (Nilssen et al., 1996).

The attractiveness of DMTS to gravid calliphorid females (Table 1) is rather puzzling because DMTS is a widespread semiochemical associated with various resources that attract flies. Among others, DMTS is a bacterial decomposition product of both vertebrate carrion (Vass, 2001; Statheropoulos et al., 2005; Dekeirsschieter et al., 2009) and faeces (Moore et al., 1987; Garner et al., 2007; Hales et al., 2012). Depending on their physiological state, blow flies will forage for faeces to obtain nutrients and seek carrion to oviposit. To discern between faeces and carrion, foraging blow flies must exploit resource-specific information. The concentration of DMTS is likely not sufficiently specific, because it will change with distance to the resource. Instead, blow flies may exploit semiochemical or visual cues (at closer range) that are specific to either resource. In addition to DMTS, faeces may have semiochemicals that are not part of carrion odour. Faeces may also have distinct visual cues. For example, fresh faeces from small mammals reflect ultraviolet light which Kestrels, *Falco tinnunculus* L., exploit to identify areas of vole abundance (Viitalaet al., 1995). In turn, the fur colour of fresh carrion may contribute to the specificity of the carrion cue complex. Coupled with DMTS, carrion-type colour cues such as dark red or black were more effective in attracting blow flies than bright colour cues such as white or yellow (Figure 7, Exps. 26, 27). The flies' preferential response to dark-coloured traps was not likely due to a particular thermographic profile of

these traps because temperature differentials between dark- and bright-coloured traps were merely a few degrees Celsius.

To localize an oviposition site, flies must integrate olfactory, visual, and mechanosensory information (Frye et al., 2003). The odour source (DMTS) was kept identical among paired test stimuli in each of experiments 20, 22, and 23, and thus did not contribute to the superior attractiveness of dark-coloured test stimuli. Similarly, all traps in experiments 22 and 23 were suspended at the same height above ground, suggesting that the relative motion (Kimmerle et al., 1996; Srinivasan & Zhang, 2004) between the images of traps and ground was rather uniform for all traps, and thus, that image motion cues did not likely affect trap discrimination by flies. Whether then flies preferred darker-coloured objects based on their spectrometric profile (colour), luminance, or contrast against background is difficult to interpret. Contrast between the perimeter of an object and its background certainly enhances the attractiveness of an object to house flies (Howard & Wall, 1998), and vertical edges help *Drosophila melanogaster* vinegar flies locate odour sources (Frye et al., 2003). Similarly, significant thermographic contrast between "hot" conifer cones and "cool" conifer foliage, helps western conifer seed bugs, *Leptoglossus occidentalis* L., locate seed resources (Takács et al., 2009). Yet, when we gauged contrast between test stimuli and background in our study, it seemed that the bright white and yellow colours provided a stronger contrast, or a more apparent vertical edge, against the green pasture background than did the brown or black colours. If so, foraging gravid blow flies in our study indeed discerned between trap colours and discriminated against bright white or yellow traps. This apparent colour preference, however, does not diminish the potential importance of object-background contrast on foraging or alighting decisions by flies. Moreover, colour preference may change depending on the specific combination of object and background colours and/or the physiological status and "motivation" of foraging flies. Any one of these variables could explain the seemingly inconclusive data on colour preference of blow flies (see Introduction).

In conclusion, DMTS is a key semiochemical cue that attracts gravid *L. serricata* to fresh incised rat carrion, and likely also to other vertebrate carrion. The attractiveness of DMTS is enhanced when coupled with dark colour cues that are typically associated

with animal fur. These findings imply that gravid flies respond to (at least) a bimodal cue complex when they seek oviposition sites.

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5.7. References

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Table 5.1. Details on experimental *Lucilia sericata*, number and duration of experimental replicates, and olfactory and visual cues tested in laboratory and field experiments

Exp. no.	Location	Olfactory cues (**C**)	Colour cues	Insects & no. replicates[1]			Time
				Protein-fed (13-day-old)	Protein-deprived (3-day-old)		
				♀	♀	♂	
Objective 1: Bioassay attraction of flies to intact and incised carrion							
1	Laboratory	**C1**: Intact rat; **C2:** Incised rat; **C3:** Empty control	○ ○ ○	10	–	–	3 hrs
Objective 2: Obtain headspace volatile (HSV) extract of attractive carrion and bioassay its attractiveness to flies							
2	Laboratory	**C1:** HSV extract of incised rat; **C2:** Solvent control	○ ○	10	–	–	5 min
Objective 4: Determine the essential component(s) in a synthetic blend of candidate HSV semiochemicals							
3	Laboratory	**C1:** SB[3d]; **C2:** Solvent control	○ ○	10	–	–	5 min
4, 10, 15	Laboratory	**C1:** SB; **C2:** Solvent control	○ ○	10	10	10	5 min
5, 11, 16	Laboratory	**C1:** SB minus acids; **C2:** Solvent control	○ ○	10	10	10	5 min
6, 12, 17	Laboratory	**C1:** SB minus alcohols; **C2:** Solvent control	○ ○	10	10	10	5 min
7, 13, 18	Laboratory	**C1:** SB minus aldehydes; **C2:** Solvent control	○ ○	10	10	10	5 min
8, 14, 19	Laboratory	**C1:** SB minus DMTS minus pyrazine; **C2:** Solvent control	○ ○	10	10	10	5 min
9	Laboratory	**C1:** SB minus DMTS; **C2:** Solvent control	○ ○	10	–	–	5 min
Objective 5: Investigate potential interaction between the key semiochemical(s) and colour cues for attraction of flies							
20	Laboratory	**C1a:** DMTS (1 ng); **C1b:** DMTS (1 ng)	○ ●	10	–	–	5 min
		C2a: DMTS (10 ng); **C2b:** DMTS (10 ng)	○ ●	10	–	–	
		C3a: DMTS (100 ng); **C3b:** DMTS (100 ng)	○ ●	10	–	–	
		C4a: DMTS (1000 ng); **C4b:** DMTS (1000 ng)	○ ●	10	–	–	
21	Field	**C1:** DMTS[e] (0.1%); **C2:** DMTS (1%); **S3:** DMTS (10%)	■ ■ ■				8 days
22	Field	**C1:** DMTS (10%); **C2:** DMTS (10%)	■ ■				4 days

23	Field	**C1:** DMTS (10%); **C2:** DMTS (10%)		6 days

[1]Each replicate tested 50 flies.

[2]Headspace volatile (HSV) extract was tested at 1 rat-hour-equivalent (the amount of volatiles given off one incised rat carrion during 1 h).

[3]The synthetic blend (SB) consisted of: Dimethyl trisulfide (DMTS, 40 ng), phenol (600 ng), phenylacetaldehyde (80 ng), (*E*)-2-octanal (60 ng), *meta*-cresol (420 ng), *para*-cresol (420 ng), guaiacol (180 ng), tetramethyl pyrazine (180 ng), nonanal (600 ng), isobutyric acid (300 ng), 2-methylbutyric acid (500 ng), butyric acid (50 ng), amyl acid (100 ng), isovaleric acid (300 ng), and hexanoic acid (300 ng).

Table 5.2. Total number of gravid female blow flies (*Lucilia sericata*, *L. illustris*, *Calliphora vicina*, *C. vomitoria*) captured in field experiments 21-23 (n = 12 each; dairy farm, Delta, BC, Canada) in Oakstem traps (Figure 7B) covered with white-, yellow-, or black-coloured paper, and baited with lures of different dimethyl trisulfide (DMTS) concentrations

	Total number of blow flies captured[1]							
	Exp. 21 DMTS (%)[2]				Exp. 22[3] Trap colour		Exp. 23[3] Trap colour	
Fly species	0	0.1	1	10	Black	Yellow	Black	White
L. sericata	0	2	9	62	53	1	74	13
L. illustris	0	0	0	3	2	0	5	1
C. vomitoria	0	0	0	0	0	0	1	0
C. vicina	0	0	0	1	0	0	2	0
Total	0	2	9	66	55	1	82	14
Mean	0	0.167	0.75	5.077	4.58	0.15	6.83	1.17
SE	0	0.167	0.494	1.54	2	0.083	2.91	0.64

[1]All flies were gravid except one dry female *C. vicina* for which gravidity could not be determined.

[2]Traps were baited with a 4-ml glass vial containing 900 µL of gelled paraffin oil with DMTS at 0, 0.1, 1.0 and 10%; trap colour: brown-red (see Figure 1b).

[3]10% DMTS lure.

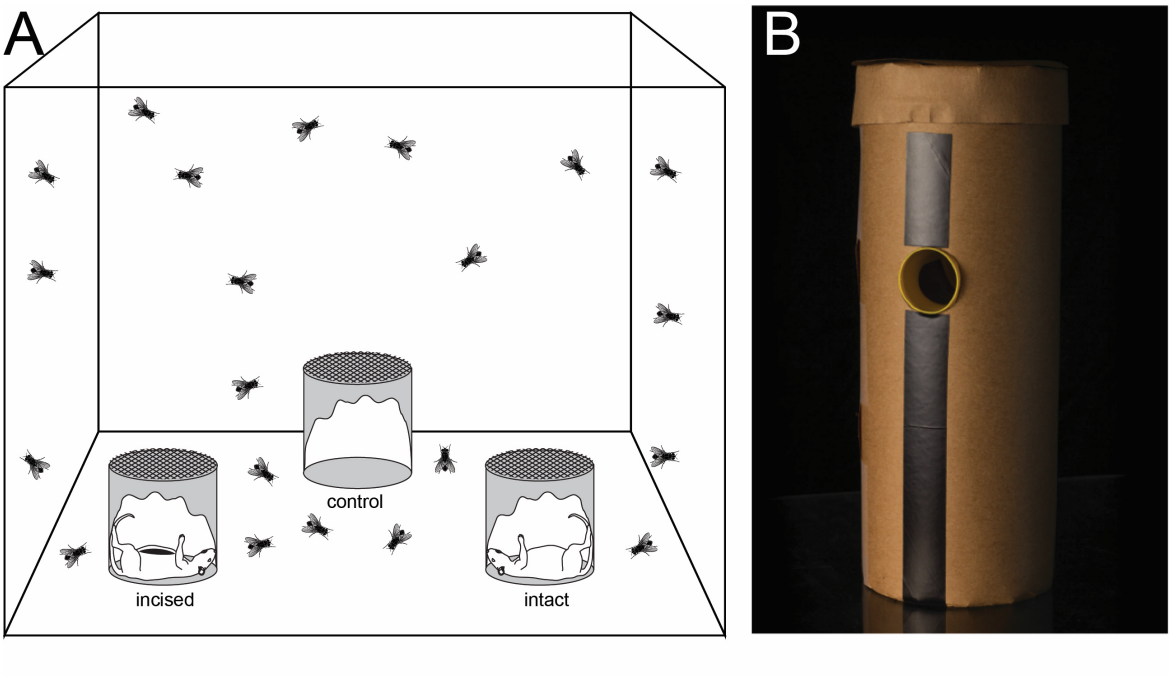

Figure 5.1. **(A) Design of two- or three-choice laboratory experiments; (B) Oak-stem trap deployed in field experiments (the depicted trap is covered in brown construction paper as one of four colours tested)**

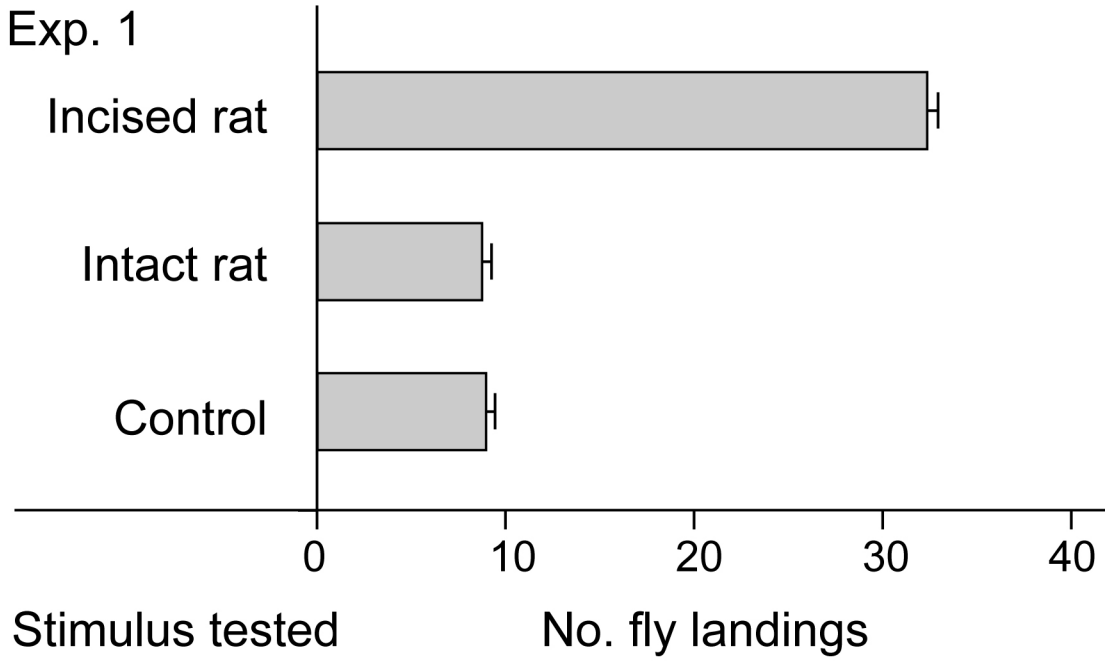

Figure 5.2. **Mean number of alighting responses by gravid female *Lucilia sericata* in experiment 1 (n = 10, 3-h duration each) on cheesecloth-covered Solo cups (see Figure 1A) that were not baited (control) or baited with either an intact rat carrion 1 h post mortem or a rat carrion 1 h post mortem incised immediately after CO_2-euthanization. The incised rat carrion received significantly more than 50% of the flies' alighting responses (one-tailed t-test, P<0.0001).**

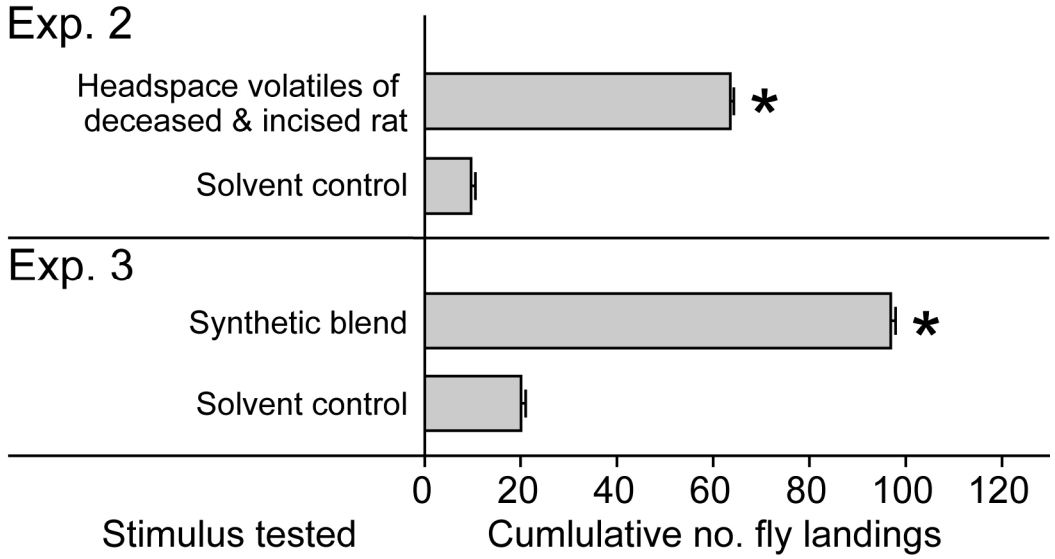

Figure 5.3. **Mean cumulative number of alighting responses by gravid female *Lucilia sericata* in experiments 2 and 3 (n = 10 each, 5-min duration each) on cheesecloth-covered Solo cups (see Figure 1A) baited with headspace volatile extract (HSV) of incised rat carrion at 1 rat-hour-equivalent (the amount of volatiles given off one incised rat carrion during 1 h) or a solvent control (Exp. 2), or to a synthetic blend of candidate semiochemicals (see Figure 4, Table 1) in the HSV extract or a solvent control (Exp. 3).**

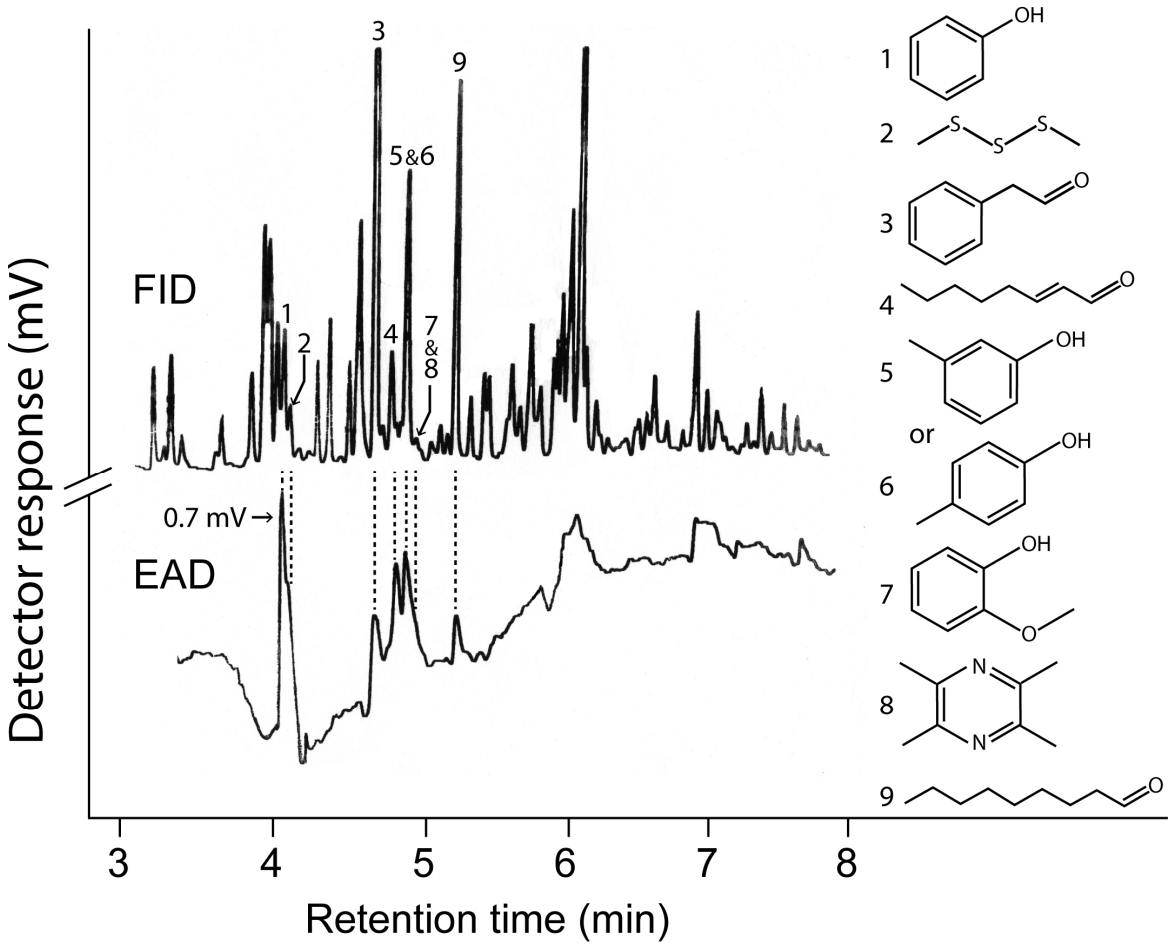

Figure 5.4. **Representative recording of the responses of a gas chromatographic flame ionization detector (FID) and an electroantennographic detector (EAD: female *Lucilia sericata* antenna) to aliquots of headspace volatile extract of incised rat carrion. Nine components elicited antennal responses, as follows: 1) phenol, 2) dimethyl trisulfide, 3) phenylacetaldehyde, 4) (*E*)-2-octanal, 5,6) *meta*-cresol and/or *para*-cresol, 7) guaiacol, 8) tetramethyl pyrazine, and 9) nonanal.**

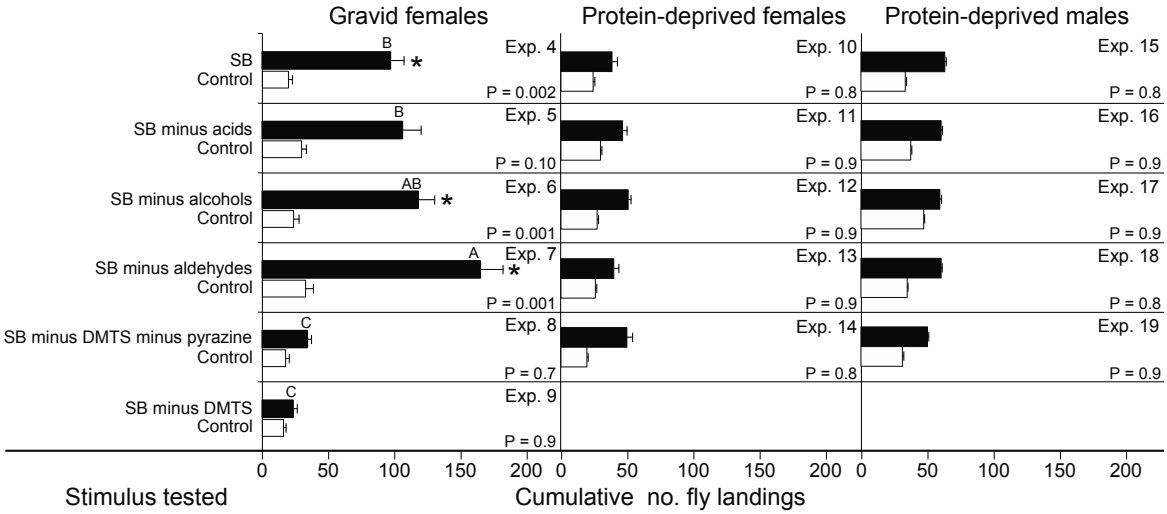

Figure 5.5. Mean cumulative number of alighting responses by *Lucilia sericata* blow flies in experiments 4-19 (n = 10 each, 5-min duration each) on cheesecloth-covered Solo cups (see Figure 1A) baited with a synthetic blend (SB; Table 1) of the nine components that elicited antennal responses in headspace volatile extracts of deceased and incised rat carrion (see Figure 4), or with partial blends of these components. Pentane served as a control stimulus. In each experiment, a star denotes a treatment stimulus that received significantly more than 70% of the flies' alighting responses (one-tailed t-test: P<0.001). Within each of experiments 4-9, bars with the same superscript uppercase letter are not significantly different (Tukey's HSD: P>0.05). DMTS = dimethyl trisulfide.

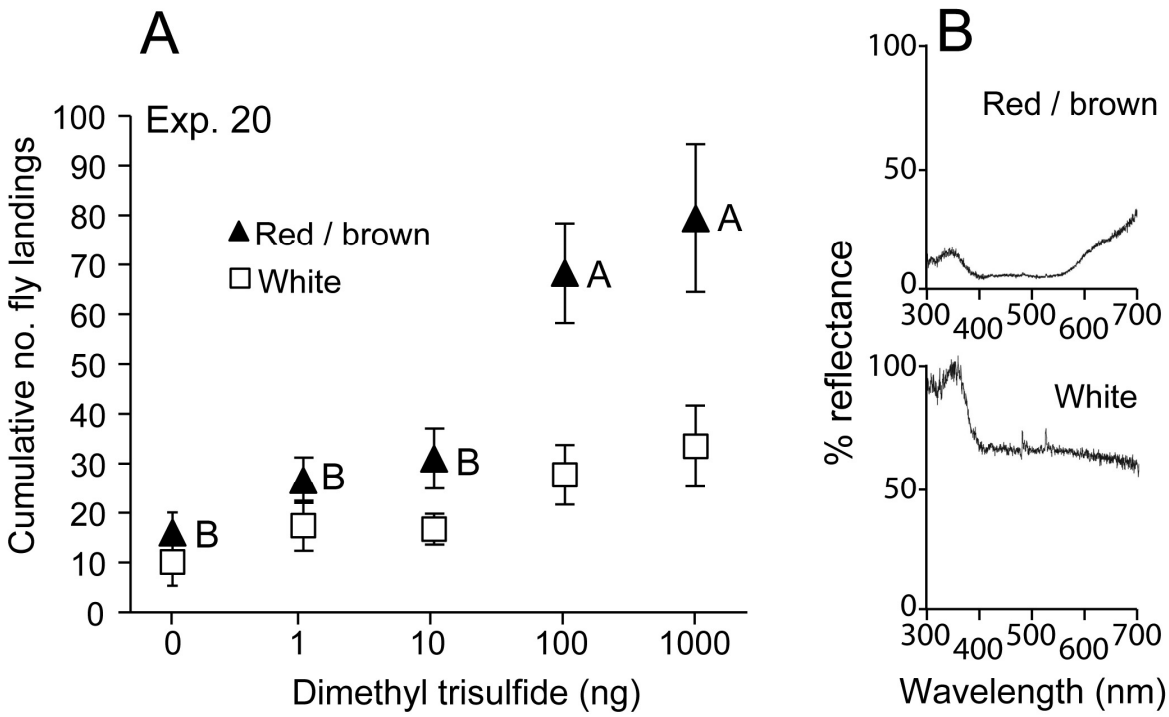

Figure 5.6. **(A) Mean cumulative number of alighting responses by Lucilia *sericata* blow flies in experiment 20 (n = 10, 5-min duration each; Table 1) on paired Solo Cups (see Figure 1A) baited with an identical amount of dimethyl trisulfide (DMTS) but covered with either a white cheesecloth (open square) or red cheesecloth (solid triangle). Triangles with the same letter are not significantly different for both DMTS concentration and DMTS concentration*cheesecloth colour (Tukey's HSD: P>0.05); (B) Spectral reflectance from white or red cheesecloth.**

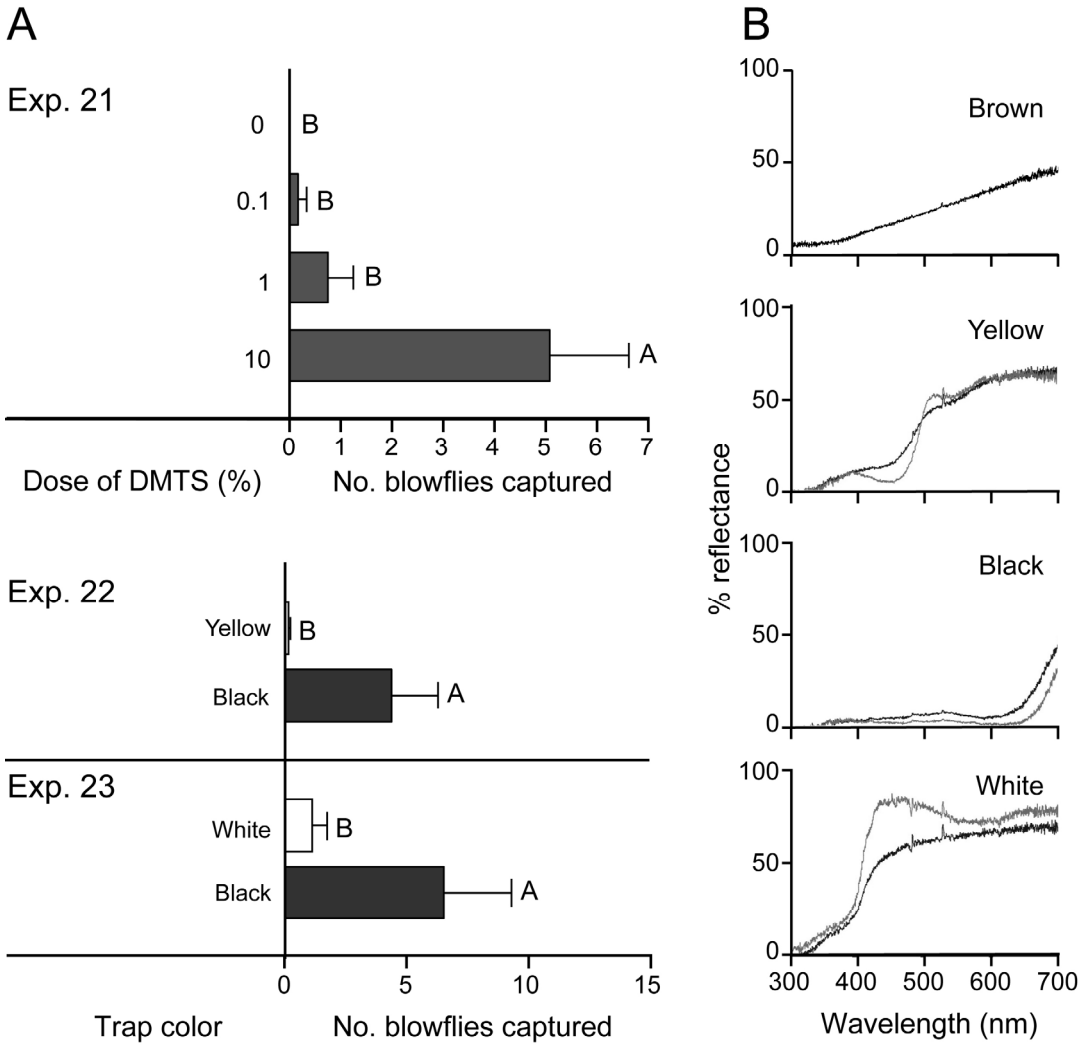

Figure 5.7. **(A) Mean number of *Lucilia sericata* blow flies captured in experiment 21 (n = 10; Table1) in red/brown Oak-stem traps (see Figure 1B) baited with different amounts of dimethyl trisulfide (DMTS), or captured in experiment 22 (n = 12) and 23 (n = 12) in traps baited with an identical amount (10%) of DMTS but covered with yellow-, white-, or black-coloured paper. In experiment 21, bars with a different letter are significantly different (Tukey's HSD: P<0.00015); (B) Spectral reflectance from red/brown, black and faded black, yellow and faded yellow, and white and faded white paper covering traps.**

Chapter 6.

Is aggregated oviposition by the blow flies *Lucilia sericata* and *Phormia regina* (Diptera: Calliphoridae) really pheromone-mediated?

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6.1. Abstract

When female blow flies *Lucilia sericata* and *Phormia regina* (Diptera: Calliphoridae) oviposit in aggregations on a carrion resource, even-aged larval offspring reportedly develop faster, and fewer are parasitized or preyed upon. The benefits of aggregated oviposition equally affect con- and heterospecific larvae sharing a resource. The benefits imply that female blow flies engage in coordinated, pheromone-mediated oviposition behaviour. Yet, repeated attempts to identify oviposition pheromones have failed invoking doubt that they exist. Simply by regurgitating and feeding on carrion, flies may produce attractive semiochemicals. If flies were to aggregate on a carrion resource in response to feeding flies rather than ovipositing flies, then the semiochemical cue(s) may be associated with the salivary gland. Working with female *L. sericata* and *P. regina* and using liver as a surrogate oviposition medium, we test the hypotheses, and present data in their support, that (1) gravid or non-gravid females ovipositing and/or feeding on liver enhance its attractiveness to gravid and non-gravid females; (2) females respond to semiochemicals from feeding heterospecific females; (3) females respond equally well to semiochemicals from feeding con- and heterospecific females; (4) macerated head tissues of females applied to liver enhance its attractiveness; and (5) females in direct

contact with liver, but not just residing next to it, enhance attraction of other flies. We conclude that oviposition site-seeking females do not respond to an oviposition pheromone. Instead, they appear to co-opt semiochemicals associated with feeding flies as resource indicators, taking chances that resources are suitable for oviposition, and that ovipositing flies are present.

6.2. Introduction

Aggregations of insects mark suitable habitat or resources (McFarlane *et al.*, 1983; Ceyto *et al.*, 1984; Rust & Appel, 1985; Sauphanor & Sureau, 1993; Walker *et al.*, 1993; Siljander *et al.*, 2007), reduce desiccation (Joosse, 1970; Joosse & Verhoef, 1974), increase mate encounters (Sexton & Hess, 1968; Joosse, 1970; Verhoef & Nagelkerke, 1977; Walker *et al.*, 1993; Woodbury & Gries 2013a,b; Woodbury *et al.*, 2013), or foster contact between juveniles which promotes their growth and development (Ishii & Kuwahara, 1967; Takeda, 1980).

Aggregated oviposition by flies (Fenton *et al.*, 1999; Lam *et al.*, 2007; Slone & Gruner, 2007; Charabidze *et al.*, 2011; Zheng *et al*., 2013) helps ameliorate resources for larval offspring. Female house flies, *Musca domestica* L. (Diptera: Muscidae), for example, preferentially oviposit near freshly deposited conspecific eggs. The many even-aged larvae warm and moisten the organic material (Bryant, 1970; Barnard & Geden, 1993) and curtail growth of competitive fungi (Zvereva, 1986). Oviposition near fresh eggs is stimulated by pheromonal and visual cues from ovipositing adults (Collins & Bell, 1996; Jiang *et al*., 2002).

Gravid female blow flies (Calliphoridae) also oviposit in aggregations. Females depend on fresh carrion as an oviposition site, which provides protein for oocyte maturation (Lee *et al.*, 1992) and nutrient resources for larval development (Ireland & Turner, 2006). Aggregated oviposition by conspecific females or even con- and heterospecific females, and concurrent development of many larvae increase the fitness of ovipositing females. In resources with many larvae, relatively fewer are preyed upon or parasitized (Wall *et al.*, 2001; Chin & Baharudin, 2009; Charabidze *et al.*, 2011; Archer & Elgar, 2003), and larvae develop more quickly by sharing digestive fluids and taking advantage of elevated resource temperatures (Charabidze *et al.*, 2011).

Vertebrate carcasses as ephemeral resources are exploited by scavenging terrestrial vertebrates, insects, fungi, and microbes (DeVault *et al.*, 2003; Horenstein *et al.*, 2012; Barton *et al.*, 2013; Pechal *et al.*, 2014). Species in the blow fly guild such as *Lucilla sericata* (Meigen) and *Phormia regina* Meigen (Diptera: Calliphoridae) reduce interspecific resource competition by responding within minutes or hours to recently

deceased animals (Kuusela & Hanksi, 1982; Archer & Elgar, 2003). Ovipositing females enhance the attractiveness of a carrion resource (Barton Browne *et al.*, 1969; Ashworth & Wall, 1994) apparently by releasing an oviposition pheromone (Barton Browne *et al.,* 1969; Ashworth & Wall, 1994; Wertheim *et al.*, 2005). However, repeated attempts to extract the oviposition pheromone from the abdomen or ovipositor of female *L. sericata,* or the surface of oviposited eggs, have failed (unpublished data), invoking doubt whether an oviposition pheromone really exists. If it does, then only gravid females should produce and respond to it. Moreover, with no evidence for specific aggregation, sex attractant or contact mate recognition pheromones in blow flies (Benziane & Campan, 1993; Stoffolano *et al.*, 1997), one might wonder how blow flies find mates or even feeding sites. Conceivably, the reproductive biology of blow flies may be linked to carrion resources. Simply by regurgitating and feeding on carrion, flies may enhance its attractiveness. These feeding flies may inadvertently attract gravid and non-gravid females and even males. Based on their sex, age and reproductive status, flies attracted to a resource may then obtain a protein meal, find a mate, or commence oviposition.

Con- and heterospecific blow flies can arrive concurrently at carrion (Archer & Elgar, 2003; Tabor *et al.*, 2005; Reibe & Madea, 2010) and larvae of several species including *P. regina* and *L. sericata* can co-develop on the same carrion resource (Smith & Wall, 1997; Tabor *et al.*, 2004). The benefits of aggregated oviposition by con- and heterospecific flies (see above; Jones & Turner, 1987; Byrd & Castner, 2001; Archer & Elgar, 2003), and the resulting co-development of their larvae, may outweigh adverse effects of interspecific larval competition for carrion resources (Denno & Cothran, 1975; Kneidel, 1984; Kouki & Hanski, 1995). Conversely, continued oviposition by con- and heterospecific flies on the same resource may lead to overcrowding of larvae and reduce the fitness of ovipositing females. Thus, gravid female flies making oviposition decisions would benefit from detecting olfactory signals or cues associated with both con- and heterospecific flies. Moreover, with no evidence for partitioning of a carrion resource during larval development, the presence of con- or heterospecific larvae would have the same beneficial or adverse effect on larval development.

There is consensus that ovipositing gravid blow flies enhance the attractiveness of a carrion resource by semiochemical signals or cues (Denno & Cothran, 1975; Kneidel,

1984; Wertheim *et al.*, 2005) but the source of these semiochemicals is not known. Studies have focused primarily on demonstrating possible evidence for an oviposition pheromone (Barton Browne et al., 1969) and the attractiveness of carrion (Hall & Doisy, 1993; Barton *et al.*, 2013). If flies were to aggregate on a carrion resource in response to feeding flies rather than ovipositing flies (see above), then the semiochemical cue(s) may be present in the vomitus or salivary secretions of flies. As early as 1955, Dethier noted that fed-on food was more attractive to blow flies than non-fed on food. Even if the flies do not signal themselves, their salivary secretions may contain enzymes and microorganisms that initiate metabolism of carrion carbohydrates, proteins or lipids (Dethier, 1955; Telford *et al.*, 2012). These metabolic activities facilitate liquefaction and breakdown of the carrion surface for fly feeding, and in the process produce volatile metabolites (Telford *et al.*, 2012; Park *et al.*, 2013; Zheng *et al.*, 2013) that are likely to attract flies. Irrespective of whether the semiochemicals are produced by the flies, their enzymes or microorganisms, they are likely associated with secretions from the salivary gland in the flies' head.

Working with *L. sericata* and *P. regina* females and using liver as a surrogate carrion oviposition medium, we tested the hypotheses that (1) gravid or non-gravid females ovipositing and/or feeding on liver enhance its attractiveness to gravid and non-gravid females; (2) females respond to semiochemicals from feeding heterospecific females; (3) females respond equally well to semiochemicals from feeding con- and heterospecific females; (4) macerated head tissues of females applied to liver enhance its attractiveness; and (5) females in direct contact with liver, but not just residing next to it, enhance attraction of other flies.

6.3. Materials and methods

6.3.1. Source of experimental flies

Colonies of *L. sericata* and *P. regina* were reared in the insectary of Simon Fraser University, starting a new colony annually from local wild type flies. Flies were kept under a L16:D8 photoperiod, at 30-40% relative humidity, and 23-25^{o} C.

Flies were aged 7-13 days prior to bioassays. Gravid flies were provided with water, sugar, and milk powder *ad libitum*, and given access to bovine liver (Supreme Meat Supplies Ltd., Burnaby, BC, Canada) for 15 min once per day. Non-gravid flies were provisioned with a similar diet except for milk powder and bovine liver.

6.3.2. General experimental design

For each experimental replicate, 50 cold-sedated gravid or non-gravid female flies were placed into a wire mesh cage (61 × 61 × 61 cm; BioQuip®, Compton, CA, USA) illuminated from above by fluorescent lights (Phillips F32TA, Amsterdam, Netherlands). These flies, termed here "response flies (RFs)", were offered two separate test stimuli randomly assigned to one of two glass Petri dishes (90 × 50 mm; Pyrex®, Massachusetts, USA) positioned on opposite sides of the cage, 30 cm apart from each other (Fig. 1a). The control stimulus was freshly sliced bovine liver (from several animals) (~100 g), and the treatment stimulus was freshly sliced liver together with 30 gravid or non-gravid female flies, termed here "stimulus flies (SFs)". To standardize visual cues, both Petri dishes were wrapped in white construction paper (Pacon®, Wisconsin, USA) and covered with white cheesecloth (2 ply, 16 × 16 cm; VWR Canlab, Edmonton, Alberta, Canada), which was secured with a rubber band (Dixon Star®, Ontario, Canada). The number of RFs present on cheesecloth of each Petri dish was counted every 5 min for 1 h and averaged. All recordings commenced within 30 min of having placed cold-sedated flies into cages, except for experiments 1 through 8, where recordings commenced at the onset of oviposition by flies.

Hypothesis 1: Gravid or non-gravid females ovipositing and/or feeding on liver enhance its attractiveness to gravid and non-gravid females

Hypothesis 1 was tested employing a full factorial design with four possible combinations: (1) gravid RFs, gravid SFs; (2) gravid RFs, non-gravid SFs; (3) non-gravid RFs, gravid STs; and (4) non-gravid RFs, non-gravid STs. In experiments 1-4 (n = 10 each) with *L. sericata,* and in experiments 5-8 (n = 12 each) with *P. regina,* test stimuli were introduced into bioassay cages, and landings of flies on the cheese cloth of test stimuli were recorded as described under general experiment design (see above).

Hypothesis 2: Females respond to semiochemicals from feeding heterospecific females

Experiment 9 (n = 12) tested the response of *L. sericata* to liver with or without 30 gravid female *P. regina,* and experiment 10 (n = 12) tested the response of *P. regina* to liver with or without 30 non-gravid female *L. sericata.* Test stimuli were introduced into bioassay cages, and landings of RFs on the cheesecloth of test stimuli were recorded as described under general experiment design (see above).

Hypothesis 3: Females respond equally well to semiochemicals from feeding con- and heterospecific females

Experiments 11 and 12 (n = 12 each) tested the response of 50 gravid female *L. sericata* (Exp. 11), or 50 gravid female *P. regina* (Exp. 12), to liver with either 30 non-gravid female *L. sericata* or 30 non-gravid female *P. regina.* Test stimuli were introduced into bioassay cages, and landings of RFs on the cheesecloth of stimuli were recorded as described under general experiment design (see above).

Hypothesis 4: Macerated head tissues of females applied to liver enhance its attractiveness

Parallel-run experiments 13-15 (n = 16 each) tested the response of 50 gravid female *L. sericata* to liver alone or to liver with topically applied macerated heads (Exp. 13), thoraces (Exp. 14), or abdomens (Exp. 15) of 30 *L. sericata* females. For each replicate, test stimuli were prepared by severing the tagmata of 30 cold-sedated gravid females, by macerating separately heads, thoraces, and abdomens, and by applying the macerated tissue onto liver. Macerated tissues of fly thoraces and abdomens were not expected to enhance the attractiveness of liver but were tested to gauge the effect of body tissue *per se*. Test stimuli were introduced into bioassay cages, and landings of RFs on the cheesecloth of test stimuli were recorded as described under general experimental design (see above).

Hypothesis 5: Females in direct contact with liver, but not just residing next to it, enhance attraction of other flies.

To ascertain whether RFs are attracted to a combination of liver and SF semiochemicals or to semiochemicals associated with SFs feeding on liver, parallel-run

experiments 16-17 (n = 10 each) were designed to test the response of 50 gravid female *L. sericata* to liver alone or to liver with 30 conspecific SFs that did (Exp. 16), or did not (Exp. 17), have access to liver (Fig. 1d,e). Access of SFs to liver was prevented through a glass insert (0.85 × 0.45 cm) within the same Petri dish housing SFs (Fig. 1d,e). Test stimuli were wrapped in construction paper and covered with cheesecloth to occlude visual cues. Test stimuli were introduced into bioassay cages, and landings of RFs on the cheesecloth of test stimuli were recorded as described under general experiment design (see above).

6.3.3. Statistical analyses

In each of two-choice experiments 1-17, the mean proportion of fly landings on test stimuli was analyzed by a two-tailed Wilcoxon signed-rank test expecting a preferred stimulus to induce >50% of the flies' alighting responses. The absolute numbers of fly landings on treatment stimuli in experiments 1-4 and 5-8 were analyzed by a standard poisson regression model using the glim mix procedure with fixed effect factors for treatment, time, and for treatment*time interaction, incorporating random blocks (cages) in the model. These analyses were followed by pairwise comparisons using the Tukey's Kramer adjustment test to determine significant differences in the flies' response to various treatment stimuli. Statistical analyses for the latter part of experiments 1-8 were run using Statistical Analysis System (SAS Institute Inc.).

In experiments 13-15 and 16-17, only the treatment effect - not time as a factor - on the flies' response was of interest. Thus, after averaging absolute numbers of fly landings in each replicate, means were analyzed with Kruskel Walis test. Statistical analyses employed JMP 10® (SAS Institute Inc.) for Mac® (Apple Inc., Cupertino, CA, USA).

6.4. Results

Hypothesis 1: Gravid or non-gravid females ovipositing and/or feeding on liver enhance its attractiveness to gravid and non-gravid females

In experiments 1-4 with *L. sericata* (Fig. 2, top), and in experiments 5-8 with *P. regina* (Fig. 2, bottom), test stimuli comprising liver plus gravid or non-gravid SFs received significantly more than 50% of the flies' landing responses in all experiments except 8, indicating that SFs irrespective of their physiological state feeding on liver were more attractive to RFs than liver alone. Results of statistical analyses of all eight experiments are reported in figure 2.

In experiments 1-4 with *L. sericata*, the treatment (SFs or RFs being gravid or non-gravid) had no significant effect on the flies' response ($F_{3,32}$ = 2.75, P = 0.058; Fig. 2 top), but there was a significant effect of time ($F_{11,353}$ = 4.04, P < 0.0001) and treatment*time ($F_{33,\ 352}$ = 1.48, P = 0.046). The significant effects of time and treatment*time are due to variation in the flies' response between some recording intervals but are not due to a consistent increase or decrease in response over time (Fig. 3, top).

In experiments 5-8 with *P. regina*, the treatment (SFs or RFs being gravid or non-gravid) had a significant effect on the RFs' response ($F_{3,43}$ = 3.24, P = 0.0312, Fig. 2, bottom). For example, fewer non-gravid RFs than gravid RFs responded to non-gravid SFs on liver (Exps. 6, 8). There was no significant effect of time ($F_{11,473}$ = 1.73, P = 0.064) and treatment*time ($F_{33,\ 473}$ = 1.42, P = 0.065; Fig. 3, bottom).

Hypothesis 2: Females respond to semiochemicals from feeding heterospecific females

The test stimulus comprising liver plus non-gravid female *P. regina* received significantly more than 50% of the landing responses by gravid female *L. sericata* (W = 33, df = 11, P = 0.001; Fig. 4, Exp. 9), indicating that female *L. sericata* respond to semiochemical cues derived from heterospecific females. Similarly, the test stimulus comprising liver plus non-gravid female *L. sericata* received significantly more than 50% of the landing responses by gravid female *P. regina* (W = 39, df = 11, P = 0.0005; Fig. 4,

Exp. 10), indicating that female *P. regina* respond to semiochemical cues derived from heterospecific females.

Hypothesis 3: Females respond equally well to semiochemicals from feeding con- and heterospecific females

When bioassaying the stimulus of liver plus non-gravid female *P. regina* versus that of liver plus non-gravid female *L. sericata* for the response of gravid female *L. sericata* (Exp. 11), or gravid female *P. regina* (Exp. 12), the response to conspecific SFs did not significantly differ from 50% of the flies' landing responses in each experiment [Exp. 11 (*L. sericata* RFs): $W = 30.5$, df = 14, $P = 0.086$; Exp. 12 (*P. regina* RFs): $W = 6.5$, df = 14, $P = 0.703$; Fig. 5]. Based on these statistical analyses, neither *L. sericata* females nor *P. regina* females prefer semiochemical cues from conspecifics to those from heterospecifics.

Hypothesis 4: Head tissues of females applied to liver enhance its attractiveness

Each of the stimuli comprising liver plus macerated tagma tissue of *L. sericata* (head tissue: Exp. 13; thorax tissue: Exp. 14; abdomen tissue: Exp. 15) received significantly more than 50% of the flies' landing responses (Fig. 6), indicating that semiochemicals derived from any tagma tissue rendered liver more attractive to flies than liver alone. This tissue-related effect did not differ among the three tagmata tested ($F_{2,45} = 1.192$, $P > 0.313$).

Hypothesis 5: Females in direct contact with liver, but not just residing next to it, enhance attraction of other flies

The stimulus of liver with liver-feeding SFs (S1) did receive significantly more than 50% of the flies' landing responses (Exp. 16, Fig. 7), whereas the stimulus of liver with SFs being physically separated from it (S2) did not (Exp. 17, Fig. 7). S1 was significantly more attractive to gravid RFs than was S2 ($F_{1,20} = 43.858$, $P < 0.0001$), indicating that attractive semiochemicals were associated with feeding SFs.

6.5. Discussion

Our data support the conclusion that (1) gravid and non-gravid females of both *L. sericata* and *P. regina* present on an oviposition site enhance its attractiveness to gravid and non-gravid conspecific females; (2) female *L. sericata* respond to semiochemicals from *P. regina* and *vice versa*; (3) semiochemicals from female *L. sericata* and *P. regina* are equally attractive to both female *P. regina* and *L. sericata*; (4) macerated head, thorax or abdomen tissue of gravid female *L. sericata* is equally effective in enhancing the attractiveness of an oviposition site to gravid female *L. sericata*; and (5) *L. sericata* females in direct contact with liver, but not just residing next to it, enhance attraction of other flies.

It has been hypothesized that gravid female blow flies produce an oviposition pheromone (Browne *et al.*, 1969; Hammack, 1992; Wertheim *et al.*, 2005; Carlson & Mihok, 2007; Wicker-Thomas, 2007). If we were to consider an oviposition pheromone a communication signal *sensu* Marler (1967), then we should be able to demonstrate intent by the signaller, a behavioural response from the receiver, and adaptive benefits to the signaller and receiver. Although our data demonstrate attraction of flies to other flies on liver (Fig. 2), and attracted flies would benefit by feeding or ovipositing on that liver, it is not possible to rationalize intent to signal for non-gravid flies. With immature oocytes, non-gravid females would not accrue benefits from recruiting and inducing oviposition by gravid females. In turn, that gravid females are attracted to semiochemicals from groups of both gravid and non-gravid females (Fig. 2) implies that oviposition site-seeking females do not respond to an oviposition pheromone. Instead, they appear to co-opt semiochemicals associated with feeding flies as resource indicators, risking that resources are suitable for oviposition, and that flies are indeed in the process of ovipositing. Moreover, data in Figure 3 also do not support the oviposition pheromone hypothesis. If gravid females were to produce an oviposition pheromone, then the attractiveness of the liver resource should have differed in accordance with the occurrence of oviposition events or the number of flies ovipositing, but neither was the case.

Carrion is a typical oviposition site for blow flies. It is a vital but ephemeral resource for the development of blow fly larvae (Lee *et al*., 1992; Ireland & Turner, 2006). Gravid female flies must locate fresh carrion quickly and exploit it efficiently to outcompete resource competitors such as scavenging terrestrial vertebrates, other insects, fungi and microbes. Female blow flies respond within minutes to hours to the earliest stages of carrion decay (Anderson & VanLaerhoven, 1996; Archer & Elgar, 2003, DeVault *et al*., 2003; Tabor *et al*., 2005). They are attracted to carrion-derived long-range semiochemicals such as dimethyl trisulphide (Nilssen *et al.,* 1996; Aak & Knudsen, 2011; Frederickx *et al.*, 2012; Brodie *et al*., 2014). Once on the resource, the flies typically engage in aggregated oviposition, resulting in many even-aged larvae that "take over" the resource, dilute the effects of predation and parasitism (Erzinclioglu, 1996; Anderson, 2001; Archer & Elgar, 2003; Charabidze *et al*., 2011), and develop more quickly by sharing digestive fluids and taking advantage of elevated resource temperatures (Charabidze *et al.*, 2011). As the benefits of aggregated oviposition, or the disadvantage of resource crowdedness, equally affect just conspecific larvae, or con- and heterospecific larvae sharing a resource, we had hypothesized that *L. sericata* and *P. regina* females respond to their respective semiochemicals. Our data support this hypothesis. These data show that both *L. sericata* females and *P. regina* females prefer liver with heterospecific flies to liver alone (Fig. 4). That con- or heterospecific females on liver are almost equally attractive to foraging flies (Fig. 5) further supports the hypothesis of interspecific semiochemical recognition and attractiveness.

Macerated tissue of female *L. sericata* heads, thoraces and abdomens topically applied to liver were equally effective in enhancing the attractiveness of liver to foraging *L. sericata* females (Fig. 6). These results too do not support the hypothesis of an oviposition pheromone as the semiochemical attracting flies because pheromones are typically produced by a specific pheromone gland located in a specific tagma (e.g., Schauer *et al.,* 2012; Ban *et al.*, 2013). However, one common constituent of all three tagmata tested in experiments 13-15 was digestive fluid or microorganisms of the alimentary canal or digestive system. The nutrients are broken down (Boumba *et al.*, 2008) and in the process produce volatile metabolites that might have attracted the flies. It follows that stimulus flies in experiments 1-12 likely regurgitated digestive fluids onto liver to suck up the resulting liquefied and partially digested nutrients, and that they

thereby rendered such liver more attractive to foraging flies. To unequivocally show that it is not just the combination of liver and fly semiochemicals, but the feeding of flies on liver, that strongly recruits foraging flies to liver, we tested stimuli comprising liver and flies with or without access to liver. The results (Fig. 7) indicate that the enhanced recruitment effect is due to the feeding activity of flies on liver.

In conclusion, aggregated oviposition by female *L. sericata* and *P. regina* on carrion is not likely mediated by oviposition pheromones. This conclusion is based on data indicating that not only gravid but also non-gravid flies respond to semiochemicals from gravid or non-gravid flies feeding or ovipositing on a resource. Such indiscriminate response is not expected if the semiochemicals had a true signal function and were to recruit flies to an oviposition event. Instead, foraging flies appear to co-opt semiochemicals associated with feeding flies as a resource indicator. Once arrived on the resource, these flies then feed, mate, or oviposite in accordance with their sex, age and reproductive status or the properties of the resource.

6.6. Acknowledgements

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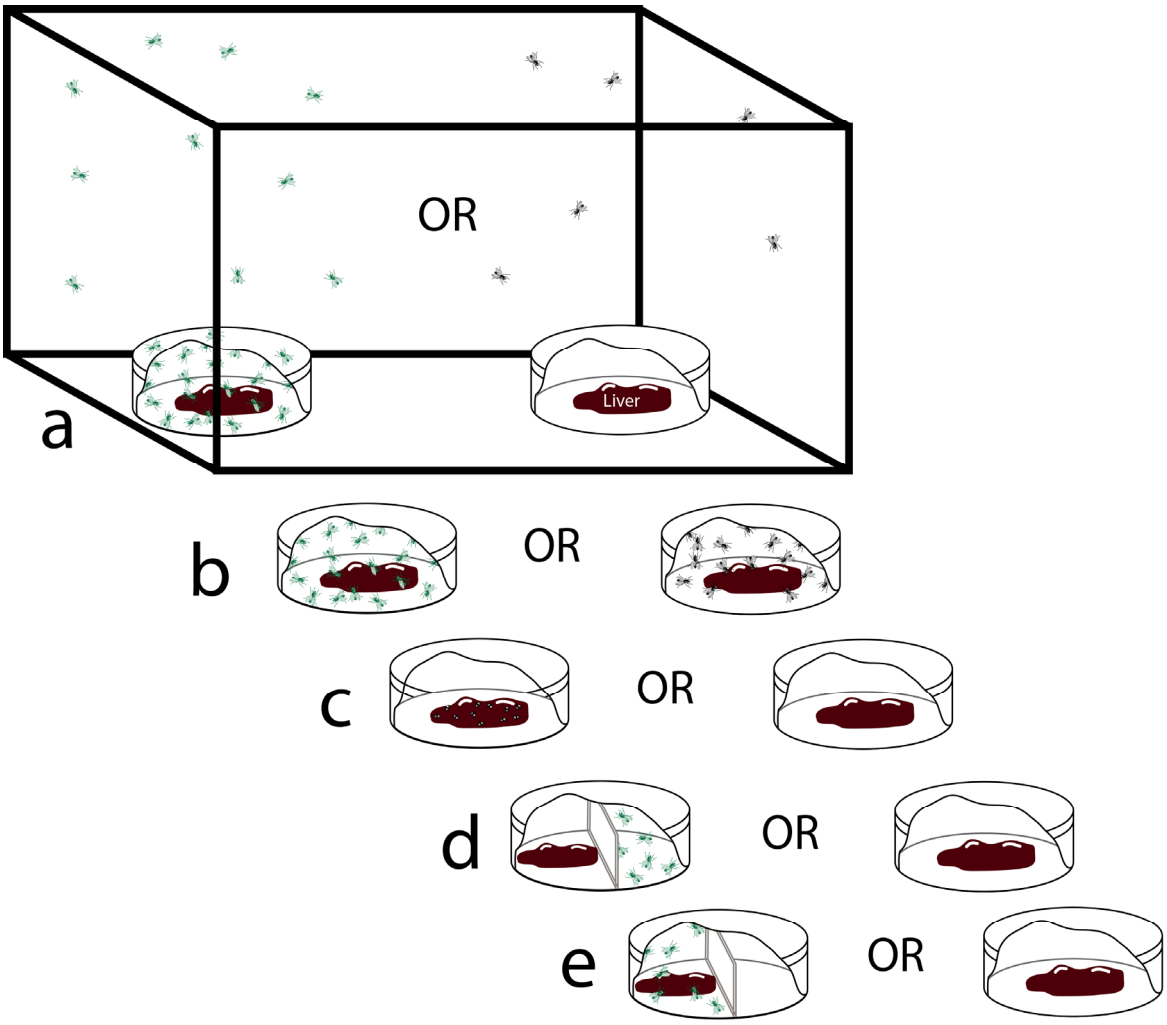

Figure 6.1. **Illustration of the general experimental design depicting a subset of 50 gravid or non-gravid female flies [(*Lucilia sericata* (black) or *Phormia regina* (green)], responding to paired test stimuli consisting of (a) liver *versus* liver plus 30 gravid or non-gravid female flies (*L. sericata* or *P. regina*), (b) liver plus 30 gravid female *L. sericata versus* liver plus 30 gravid female *P. regina,* or (c) liver *versus* liver with topically applied macerated head, thorax, or abdomen tissue of 30 gravid female *L. sericata*, (d) liver *versus* liver and flies, flies separated from liver with a glass slide, (e) liver *versus* liver and flies, flies together with liver on same half. Test stimuli were placed in Petri dishes which were wrapped in white construction paper and covered with white cheese cloth to standardize visual cues.**

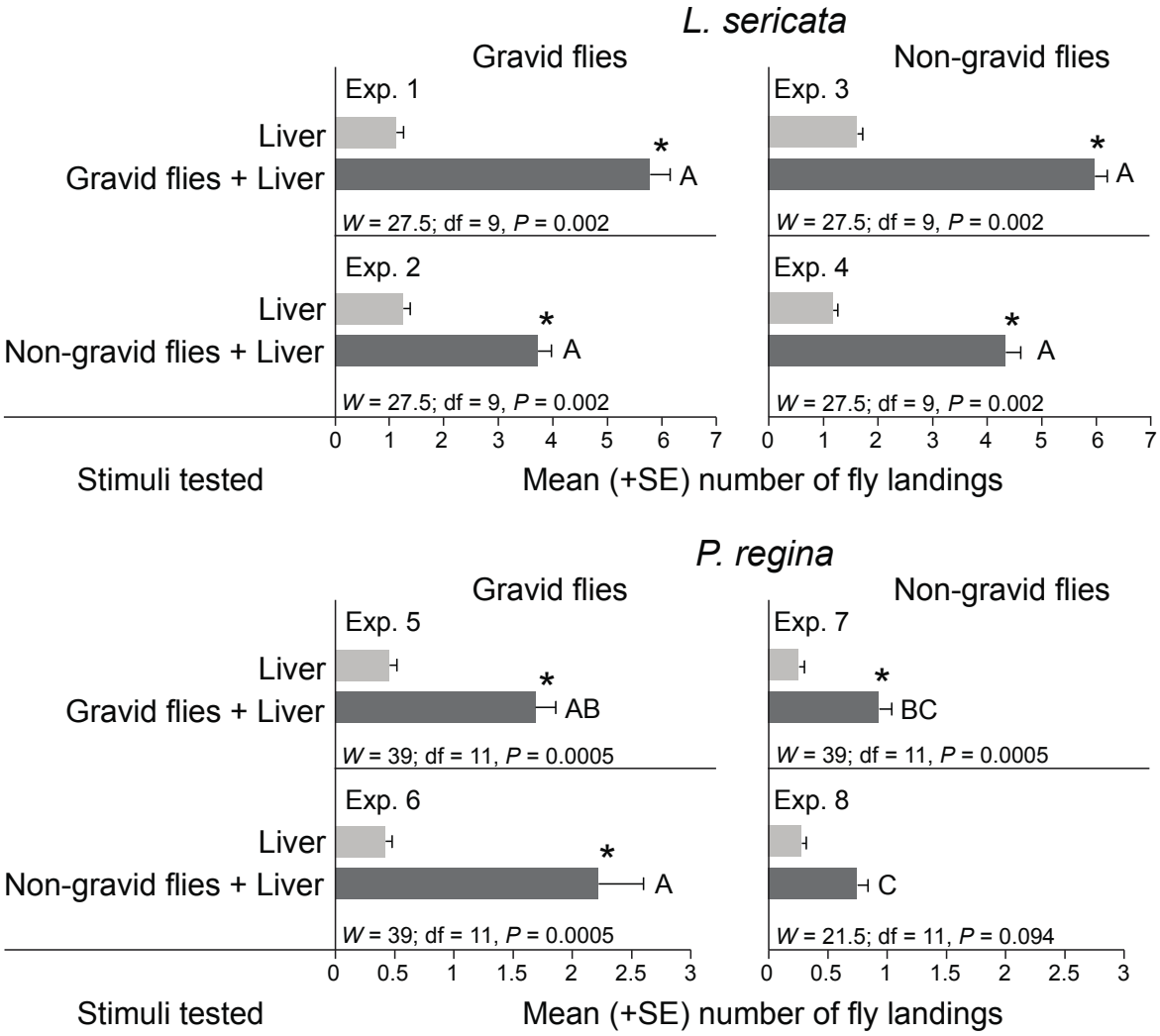

Figure 6.2. **Mean (± SE) number of alighting responses by 50 gravid or non-gravid female flies [*Lucilia sericata* (top), *Phormia regina* (bottom)] to paired test stimuli (see Figure 1) consisting of liver or liver plus 30 gravid or non-gravid conspecific females. An asterisk (*) on a paired bar indicates that the respective test stimulus received more than 50% of the females' alighting responses (two-tailed Wilcoxon signed-rank test, $\alpha = 0.05$). In each set of experiments 1-4 or 5-8, bars with the same letter superscript are statistically identical (Poisson regression model followed by Tukey's Kramer adjustment test ($\alpha = 0.05$).**

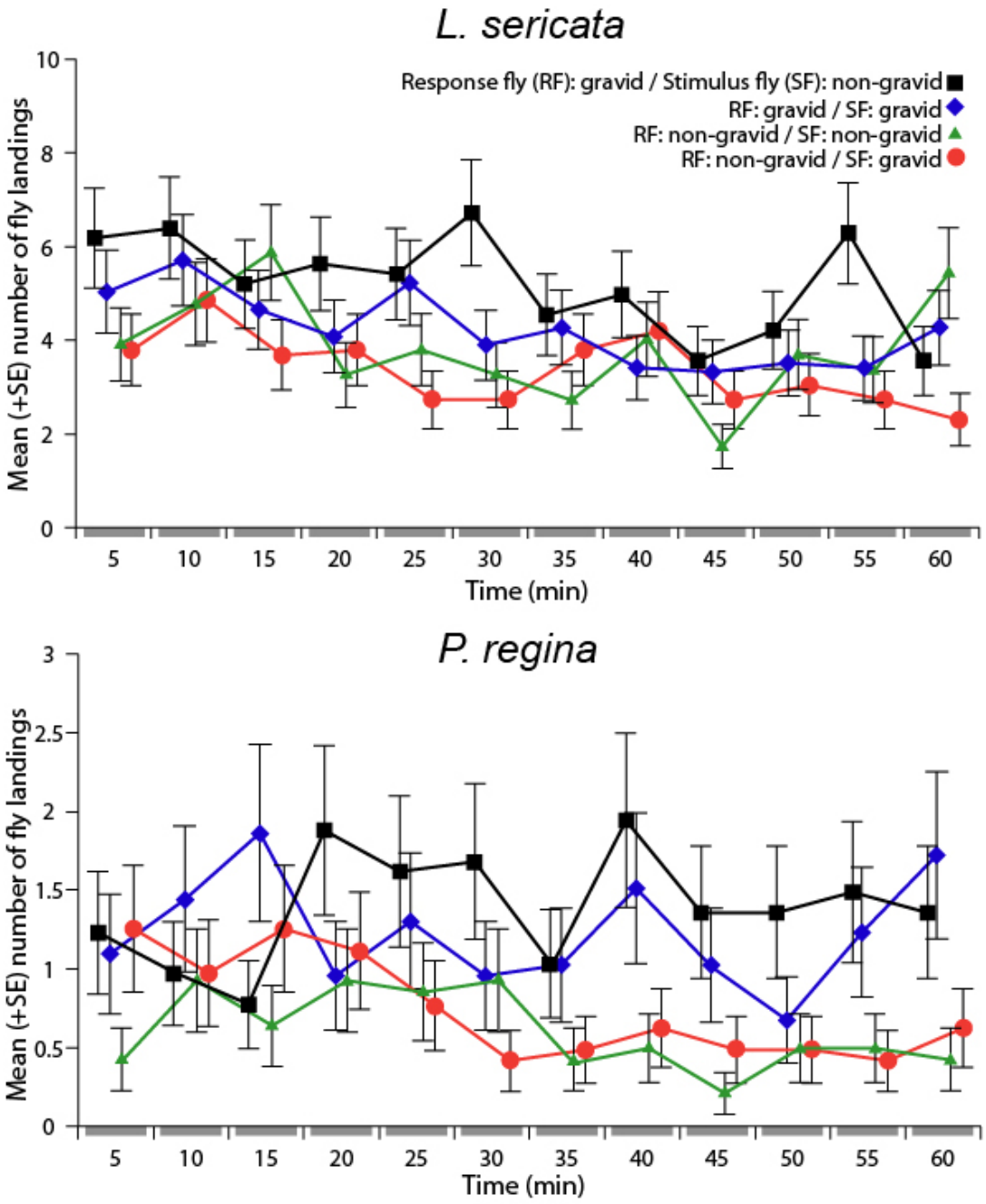

Figure 6.3. **Mean (± SE) number of alighting responses over time by gravid or non-gravid female flies [*Lucilia sericata* (top, Exps. 1-4); *Phormia regina* (bottom, Exps. 5-8)] on paired test stimuli (see Figure 1) consisting of liver plus gravid or non-gravid conspecific female flies (= stimulus flies).

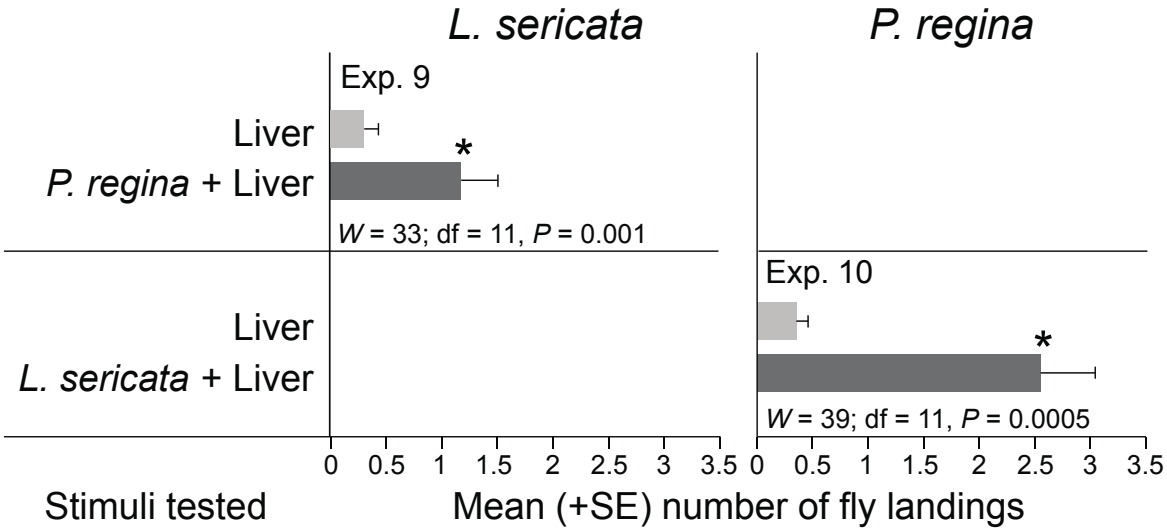

Figure 6.4. **Mean (+ SE) number of alighting responses by 50 gravid female *Lucilia sericata* or *Phormia regina* on paired test stimuli (see Figure 1) consisting of liver alone or liver plus 30 gravid conspecific females. An asterisk (*) on a paired bar indicates that the respective test stimulus received more than 50% of the females' alighting responses (one-tailed Wilcoxon signed-rank test, $\alpha = 0.05$).**

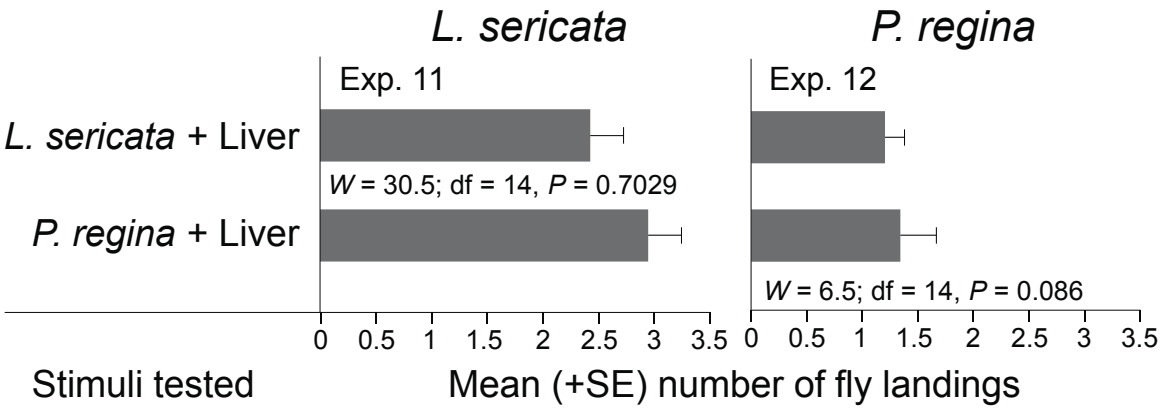

Figure 6.5. **Mean (+ SE) number of alighting responses by 50 gravid female *Lucilia sericata* or *Phormia regina* on paired test stimuli (see Figure 1) consisting of liver plus gravid con- or heterospecific females. An asterisk (*) on a paired bar indicates that the respective test stimulus received more than 50% of the females' alighting responses (one-tailed Wilcoxon signed-rank test, $\alpha = 0.05$).**

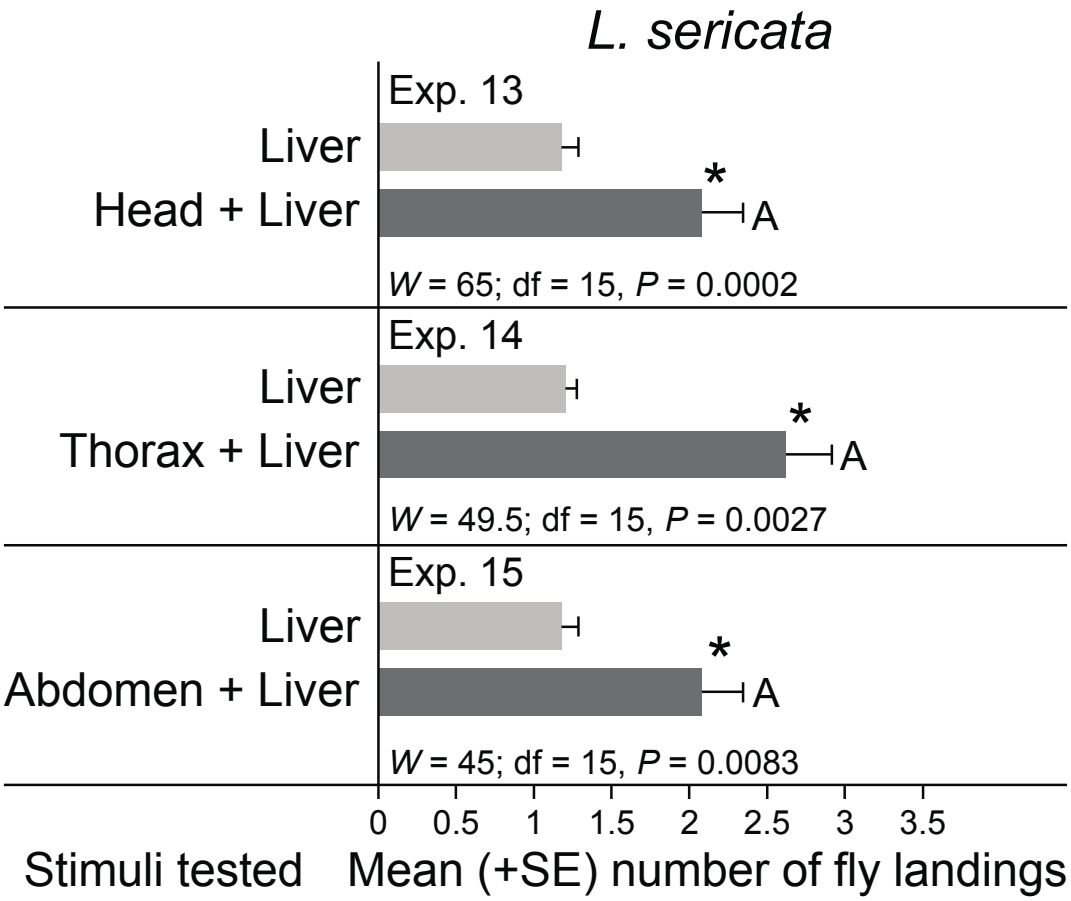

Figure 6.6. **Mean (+ SE) number of alighting responses by gravid female *Lucilia sericata* on paired test stimuli (see Figure 1) consisting of liver and liver plus macerated head tissue (Exp. 13), thorax tissue (Exp. 14) or abdomen tissue (Exp. 15) of 30 cold-sedated female *L. sericata*. An asterisk (*) on a paired bar indicates that the respective test stimulus received more than 50% of the females' alighting responses (one-tailed Wilcoxon signed-rank test, $\alpha = 0.05$). Bars with the same letter superscript are not statistically different ($F_{2,45} = 1.192$, $P > 0.313$).**

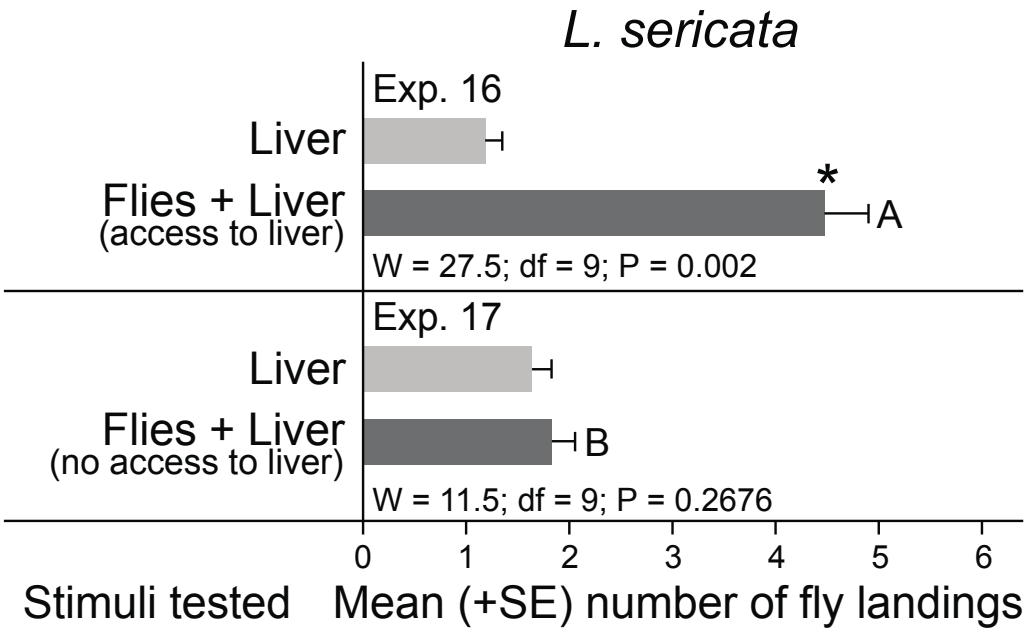

Figure 6.7. **Mean (+ SE) number of alighting responses by gravid female *Lucilia sericata* on paired test stimuli (see Fig. 1d) consisting of liver or liver plus 30 gravid female *L. sericata* that did (Exp. 16), or did not (Exp. 17), have access to liver. In experiment 16, the asterisk (*) indicates that the respective test stimulus received more than 50% of the females' alighting responses (one-tailed Wilcoxon signed-rank test, $\alpha = 0.05$). Bars with different letter superscript are statistically different ($F_{1,20} = 43.858$, $P < 0.0001$).**

Chapter 7.

Concluding summary

For this chapter, I summarize in bullet form my major findings and highlight their implications.

- Foraging for nectar and pollen, *Lucilia sericata* respond to floral scent and colour cues (particularly yellow and UV-light), with floral scent enhancing the attractiveness of floral colour.
- Pollen with adequate moisture content facilitates oocyte maturation of *L. sericata* females, implying that pollen nutrients can serve as an alternate or supplement to animal feces nutrients.
- My data support the conclusion that pollen appears to play a significant role in the foraging ecology of *L. sericata* which, in turn, may play a significant role as pollinators.

- Resource-seeking female *L. sericata* make decisions in accordance with their ontogenic development and physiological state.
- Young, protein-hungry females respond to feces and aged carrion (both food resources), whereas protein-fed, gravid females with mature oocytes respond only to fresh carrion (oviposition resource).
- Protein-hungry females are attracted to the feces semiochemicals indole in combination with one or more of the alcohols phenol, *m*-/*p*-cresol and 1-octen-3-ol.
- Gravid females distinguish between fresh carrion (oviposition resource) and aging carrion (food resource) by discriminating against carrion as soon it begins to produce appreciable amounts of indole, which is one of most abundant semiochemicals in fresh canine feces.
- My data support the conclusion that indole serves as an indicator of a food

resource rather than an oviposition resource.

- Male *L. sericata* pursue prospective mates when they move their wings.
- As thin-film reflectors, moving wings under direct light produce light flashes that attract males.
- These light flashes are absent under diffuse light, which may explain the low mating propensity of *L. sericata* on cloudy days.
- Wings also produce stable structural colours, and UV- and polarized-light-reflections, but these optic effects *per se* are insufficiently gender-specific and thus do not appear to serve as mate recognition cues.
- My data support the conclusion that mate-location and -recognition in *L. sericata* is mediated by visual rather than semiochemical cues.

- Oviposition site-seeking, gravid *L. sericata* females are more strongly attracted to incised rat carrion than to intact carrion.
- This attraction is mediated entirely by dimethyl trisulfide (DMTS).
- Increasing amounts of DMTS attract increasingly more flies.
- DMTS coupled with carrion-type colour cues (dark red, black) is more effective in attracting *L. sericata* than DMTS coupled with bright (floral) colour cues (white, yellow).
- The combination of DMTS with carrion-type colour cues attracts almost exclusively blow flies that are gravid.
- My data support the conclusion that DMTS and dark colour represent a bimodal cue complex that signifies suitable oviposition sites to gravid calliphorid flies, particularly *L. sericata*.

- Studying the underlying mechanisms of aggregated oviposition by *L. sericata* and *P. regina,* I provide evidence that oviposition site-seeking *L. sericata* females do not respond to an oviposition pheromone.
- Instead, *L. sericata* females appear to coopt semiochemicals associated with

feeding flies as resource indicators, taking chances that resources are suitable for oviposition, and that ovipositing flies are present. This conclusion is based on my data that gravid or non-gravid females ovipositing and/or feeding on oviposition resources enhance their attractiveness to gravid and non-gravid females.