CHEMICAL AND NUTRITIONAL ECOLOGY OF LUCILIA SERICATA (MEIGEN) (DIPTERA: CALLIPHORIDAE) AS RELATED TO VOLATILE ORGANIC COMPOUNDS AND ASSOCIATED ESSENTIAL AMINO ACIDS

A Dissertation

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ABSTRACT

This will be the first research aiming to investigate the chemical and nutritional ecology of *Lucilia sericata*, (Meigen) (Diptera: Calliphoridae) focusing on 1) carrion resource associated microbial volatile organic compounds (MVOCs) emissions as insect semiochemicals in regulating the olfactory response of adult *L. sericata* of different biological states, 2) the impact of exposure to these VOCs on the life history related traits of *L. sericata* adult flies, and 3) effect of carrion associated nutritional resources on blow fly larval performance.

One GFP producing *Providencia* was successfully constructed to be used to visualize bacteria along the alimentary canal of *L. sericata* larvae to implement investigation of its impact on the physiology of both *L. sericata* immature and adults, such as the immature developmental stages, the adult's potential choice for food location and oviposition. Microbes play an important role in the decomposition processes of carrion associated resources producing volatile compounds (VOCs). *L. sericata* may use MVOCs as cues to exploit the carrion resource for food, oviposition, mate etc. *Proteus* and *Providencia* spp. are among the microbes that break down as well as synthesize the essential amino acids which are the origin of MVOC. Four bacterially produced VOCs, namely DMDS (Dimethyl disulfide), indole, PAA (Phenylacetic acid), and isobutylamine, which are associated with decomposing carrion were used to investigate the olfactory response of *L. sericata* of different sexes and physiological status to study the regulation of *L. sericata* behaviors by MVOCs in exploitation of carrion resource.

Attraction and colonization of carrion resource by adult flies are followed by the subsequent development of offspring. Different responses of adult flies to volatile compounds from carrion may reflect larval preferences and performance associated with different carrion resources where they obtain nutrition and develop. In order to better understand the consequences of adult resource site selection on larval performance on the chosen carrion resource, a series of imbalanced diets deficient in essential amino acids of methionine, valine, tryptophan and phenylalanine were used to study larvae performance on carrion associated resource in term of food preferences and development. In order to investigate volatile cues sensing and physiology in *L. sericata*, DMDS was used to investigate whether the exposure to the volatile cues will also be involved in the regulation of physiology of *L. sericata* adult in terms of their life span, and defecation.

DEDICATION

I dedicate my thesis to my parents: Qiao Liu and Qin Xia, and my sister Yaqi Liu.

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NOMENCLATURE

GFP	Green fluorescent protein
E/S	Excretion and secretion
QS	Quorum sensing
VOCs	Volatile organic compounds
MVOCs	Microbial volatile organic compounds
DMDS	Dimethyl disulfide
PAA	Phenylacetic acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
GLC	Head-space gas-liquid chromatography
DEET	N, N-diethyl-m-toluamide
DNase	Deoxyribonuclease
AG	Age group
NG	Non-gravid female
GF	Gravid female

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Lucilia sericata: a model blow fly species

The green blow fly, *Lucilia sericata*, (Meigen) (Diptera: Calliphoridae) is a cosmopolitan species of great medical, veterinary and forensic importance [1, 2]. *L. sericata* is holometabolous with egg, larval, pupal and adult stages. *L. sericata* lay eggs (2,000–3,000 over lifetime) in moist places associated with appropriate resources on necrotic tissue on live and dead vertebrates [3]. Eggs hatch within 18–24 h depending on the conditions experienced [4]. Larval development is affected by abiotic (i.e., ambient temperature, humidity and food varieties) [5] and biotic variables (i.e., metabolic heat generated within a maggot mass) [5, 6]. Larvae feed for 4–5 d, molting twice as they increase in size to approximately 5–15 mm [7]. Later third instar larvae are non-feeding and typically disperse from the food source to pupate for 6 to 14 d before adult emergence [8]. Adults usually lay eggs approximately one week after emergence [9]. Their total life cycle takes two to three weeks but can be less during warmer temperatures [10, 11].

Forensic entomologists use the development of immature collected from remains to estimate a minimum postmortem interval (m-PMI) [12, 13]. However, immature development can vary due to a variety of factors. For example, *L. sericata* larvae from multiple populations and raised under controlled laboratory conditions exhibited different growth rates which was attributed to genetic variation between the populations

and environment conditions such as food, moisture, pupation substrate [14-16]. However, little is known about the microbial effect on *L. sericata* immature development.

Flies are responsible for the dispersion of over 100 pathogens into a surrounding environment that result in potentially millions of food borne illness [17]. *L. sericata* is one of the vectors of many human and animal pathogens. Adults and larvae carry pathogens in their body after consuming material from contaminated sources, such as animal carcasses and feces [18, 19]. Adult flies dispersing from one resource contaminate other locations when they land and regurgitate on them [18]. Examples of pathogens known to be dispersed by blow flies include *Mycobacterium avium* subsp. *paratuberculosis* (*M.a. paratuberculosis*) [20] and H5N1 Avian Influenza Virus [18]. Both larvae and adults of *L. sericata* can transmit pathogens such as *Mycobacterium avium* sub *avium, M.a. paratuberculosis*, and *Mycobacterium avium hominissuis* [21].

L. sericata deposit eggs in wounds of humans and animals, as well as in processed food, such as meat, fish, as well as in animal corpses, and excrement [22]. Infestations of *L. sericata* on humans in North America, Africa, and Asia have been reported, and these infestations are known to result in significant welfare and economic damage to people and associated animals [23]. For example, hospital-acquired myiasis is largely due to infestations of wounds or lesions on patients by fly larvae [22]. *L. sericata* is also notorious for infesting living tissues associated with non-human animals such as sheep [24]. Cutaneous myiasis of sheep caused by *L. sericata* has been reported in northern Europe, and a large number of sheep are infested and die of “sheep strike" each

year in England and Wales [25-27]. Although *L. sericata* affects sheep production, it is of lesser impact in Australia than its relative *Lucilia cuprina* (Weidemann) (Diptera: Calliphoridae) [28].

The use of larvae to heal wounds resulted from the observations of the curative effects of larval infestations in the wounds of injured soldiers during battles in early 20th century [29]. This cautiously induced myiasis, aiming to balance the positive effects (benign myiasis) with potential negative effects (malign myiasis) to treat the wounds is what is referred to as maggot therapy [24]. While this method was extensively used during the 1930s in the United States [30, 31], it became obsolete with the introduction of antibiotics and surgical debridement [32]; however, maggot therapy was revived during the mid-1990s due to issues with antibiotic resistance. Presently, *L. sericata* is the species most commonly used in maggot therapy [24, 33]. Approximately 15,000 patients in Europe are treated annually [34]. Its popularity is due to it (i) debriding necrotic tissue only; (ii) eliminating/ inhibiting pathogenic microbes growth in the wound by antibacterial agents in secretions; and (iii) stimulating healing by tissue remodeling [24, 34, 35]. Some researcher indicated maggot therapy to be poorly effective in patients with wounds infected with Gram-negative bacteria, such as *Pseudomonas aeruginosa*, and Gram-positive bacteria, such as *Staphylococcus aureus* [36], others however have identified antimicrobial characters of maggot excretions/secretions (ES) in healing wounds [37].

The molecules contained in the ES of the maggots are considered to contribute to wound healing by removing cell debris and non-viable tissue [24], inhibiting the pro-

inflammatory responses of phagocytes [36], promoting tissue remodeling [35], antibacterial and anti-biofilm activity [34]. A wide spectrum of proteolytic enzymes was indicated to be responsible liquefying food and reducing associated Gram-positive and Gram-negative bacteria (including *S. aureus* and *Escherichia coli*) in the wounds [38-41]. These enzymes include trypsin, leucine aminopeptidase [38] and carboxypeptidases A and B which can break down the necrotic tissue into liquid form providing nutrients for the larvae [38]. Three classes of proteolytic enzymes were detected in the secretions of *L. sericata*, including serine proteinases (trypsin-like and chymotrypsin-like) with an aspartyl proteinase (effective at pH5) and a metalloproteinase (effective at pH9) with exopeptidase characteristics [38, 39]. The proteolytic digestion of extracellular matrix of the tissue can initiate tissue repair and reconstruction contributing to the chymotrypsin-like proteinase along with haemostasis, thrombosis, inflammatory cell activation [39, 41, 42].

L. sericata ES are known to have antibacterial activity against methicillin-resistant *S. aureus* (MRSA) as high as 86% [43], glycopeptides intermediate *S. aureus* (GISA) as high as 83% [36], and pathogenic *Streptococcus* sp. with MIC value of 2mg/L [44-46]. Some agents have been found to be homologous to lysozyme and transferrin found in *Drosophila* (Diptera: Drosophilidae) while some 3-proline-rich peptides are specific to *L. sericata* [47].

An antimicrobial defensin designated as “Lucifensin”, had been expressed and purified from the extracts and ES of *L. sericata* larvae [46, 48, 49]. This peptide consists of 40 amino acid residues and has activity against *Staphylococcus carnosus*,

Streptococcus pyogenes and *Streptococcus pneumoniae* (MIC 2 mg/L), as well as *S. aureus* (MIC 16 mg/L); however it had no demonstrable activity against Gram-negative bacteria [43, 44, 46, 50-52]. The agents produced by *P. mirabilis,* a commensal bacterium that has been isolated from *L. sericata,* were documented to kill other bacteria and was referred to as ‘‘mirabilicides’’ *P. mirabilis* has been isolated from larvae of the blow fly *Calliphora vicina* (*Robineau-Desvoidy*) (Diptera: Calliphoridae) [53]. These compounds have been determined to kill microorganisms, such as *Bacillus subtilis* and *Candida albicans*, present in food material or wounds [19].

The anti-bacterial factors in the ES of maggots were previously generally categorized based on the mass of the active fractions [40, 43, 54, 55]. These include 1) molecular mass <0.5 kDa with antibacterial activity against MRSA and *E. coli* [40, 43, 54, 55], and recently a novel antibiotic C10H16N6O9, registered as Seraticin, which has been recently identified as a fraction of larval secretion showed activity against pathogens including *Staphylococcus* sp., *Bacillus* sp., *E. coli*, *Pseudomonas* sp., *Proteus* sp., *Enterococcus* sp., *Enterobacter* sp., etc. [34], 2) molecular masses of 0.5–10 kDa with antibacterial activity against *S. aureus* and 3) contains those compounds with molecular mass >10 kDa and lytic activity against lyophilised *Micrococcus lysodeikticu* [44, 54, 56]. Additionally, *Bacillus subtilis* and *Klebsiella pneumoniae* were most sensitive to the antibacterial effects of ES and *P. mirabilis* and *Staphylococcus epidermidis* were the most resistant [54].

Other compounds that are potentially antimicrobial have been isolated and identified from *L. sericata* ES. Trypsin-like and chymotrypsin-like serine proteases,

metalloproteinase, and aspartyl proteinase have also been shown to be associated with wound healing by maggot treatment [39, 57, 58]. Ammonia and associated derivatives are known to be responsible for the alkinality observed in wounds treated with blow fly larvae and are suspected to maximize activity of proteases produced by the larvae [33, 47]. Bacteria can form a biofilm, which is a formed community composed of unicellular cells that stick to each other and attached to surface [34, 59]. Over 60% of chronic wound specimens taken from 77 subjects were shown to contain a biofilm [60]. Biofilm showed resistance to antimicrobial agents due to its physical and chemical structures [61]. Biofilms of *S. epidermidis*, *S. aureus* and *Pseudomonas aeruginosa* are disrupted by deoxyribonuclease (DNase) and chymotrypsin contained in the larval secretion [34, 62]. In addition, the genes coding these molecules responsible for biofilm disruption have been studied in *L. sericata* including those that encode putative anti-microbial peptides, proline-rich peptides and potential glycosylation sites which shares sequence similarities with drosomycin, a known anti-microbial peptides isolated from *Drosophila* [63, 64].

Proteus and *Providencia*: model symbiotic bacteria

Many insects harbor commensal microbes. These symbiotic associations could be acquired vertically, horizontally or from the environment [65]. *P. mirabilis* has been isolated from larvae of the blow fly *C. vicina* [53], and *Providencia* spp. were previously shown to be associated with screwworm fly, *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae) [66]. Recent research indicated that *Providencia* spp. and *P.*

mirabilis were co-isolated from the salivary gland extracts of *L. sericata* indicating the possible interkingdom interactions between these bacteria and *L. sericata* [67].

Providencia spp. are gram negative bacteria and are pathogens of many organisms, including humans and insects [68]. *Providencia stuarti* is the most common cause of catheter-related urinary tract infection followed by *Proteus mirabilis* [68, 69]. *Providencia* and *Proteus* were responsible for the formation of crystalline bacterial biofilms on urethral catheters, that are resistant to antibiotics [70]. The bacteria also produce urease which plays important roles in kidney and bladder stone formation that lead to chronic disease [71].

Proteus is characterized by its well-known quorum sensing-regulated phenotype: swarming. Quorum-sensing (QS) bacteria can produce and release chemical signal molecules whose concentration increases as a function of increasing cell-population density [72]. Detection of the accumulation of a minimal threshold concentration of QS molecules can alter gene expressions and therefore behaviors in bacteria [73].

Proteus and *Providencia* were co-isolated from *L. sericata* previous research [67], and in an attempt to study the role of *Proteus* in the ecology of *L. sericata*. Tomberlin et al. used one of the mutated strain of *Proteus* with deficient interkingdom signaling pathways (decreased swarming ability) that was previously isolated from *L. sericata* to investigate the response of *L. sericata* to the bacteria [74]. The result from the study showed that wild type *Proteus* attracted more flies and resulted in higher chances of oviposition compared to the swarming deficient *Proteus*, further analysis of the gas chromatography demonstrated that the differentially regulated chemical molecules were

produced in wild type compared to mutant type [74], indicating that the differentially regulated molecules might be potential QS molecules that are involved in regulation of fly behaviors and decision makings.

Interkingdom communication between Diptera and *Providencia/Proteus*

Proteus mirabilis is characterized by its well-known quorum sensing (QS)-regulated phenotype: swarming. QS bacteria can produce and release chemical signal molecules whose concentration increases as a function of increasing cell-population density [72]. Detection of the accumulation of a minimal threshold concentration of QS molecules can alter gene expressions and therefore behaviors in bacteria [73]. Strain of *P. mirabilis* deficient in interkindgom signaling pathways (decreased swarming ability) were less attractive to *L. sericata* adults than the wild type [67, 74]. The swarming deficient *P. mirabilis* also had a different volatile profile than the wildtype [74], also, approximately 1/3 of the mutants in the study were rescued by known blow fly attractants [67], indicating that the differentially regulated molecules might be potential QS molecules that are involved in regulation of fly behaviors and decision makings. Specifically, Dimethyl disulfide (DMDS), phenylethyl alcohol, indole and isobutylamine were the most differentially regulated VOCs, indicating their potential roles in interkingdom interactions between bacteria and *L. sericata* [74].

The cell-cell communication system shared by *P. stuartii* and insects was previously demonstrated by the inner membrane protein AarA encoded by *P. stuartii* that is required for the release of an extracellular quorum-sensing signal shared with fruit

flies *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae) [73], it demonstrated that the expression of *P. stuartii* AarA rescued wing vein development in a *D. melanogaster rho* mutant, whereas expression of fly *rho* in a *P. stuartii aarA* mutant rescued the cell communication phenotype associated with *aarA* mutation. It was based on the fact that the protein AarA is homology to RHO and they could rescue the mutant phenotypes of each other when exogenetically expressed, that it concluded that there would be potential cell to cell communication pathways shared between this bacteria and the insect.

Volatile organic compounds released by bacteria that attract insects

Microbes play an important role in the decomposition processes of carrion, feces, urine, sweat, decomposing plant material, fungi, and other ephemeral resources. The by-products of this process are usually odorous and may serve as cues for regulating attraction or repellence of insects to these resources (Figure 1) [67].

The study of the olfactory response of *L. sericata* to VOCs was initiated in order to determine the mechanisms regulating *L. sericata* attraction and colonization of sheep (i.e., sheep strike) [111, 112]. Field research demonstrated that the attraction depended on two factors, 1) the sheep themselves (S factor) and 2) putrefying material or certain products of putrefaction, such as ammonium carbonate, indole or skatole (methyl indole) associated with the sheep wool, which were indicted in whole as P factor [111]. Limited information about the VOCs responsible for flies to sheep under natural conditions was known [75]. However, based on the limited data available, many researchers suggested

bacterial action in the wool were responsible for the production of the VOCs involved in attraction or/and oviposition [76].

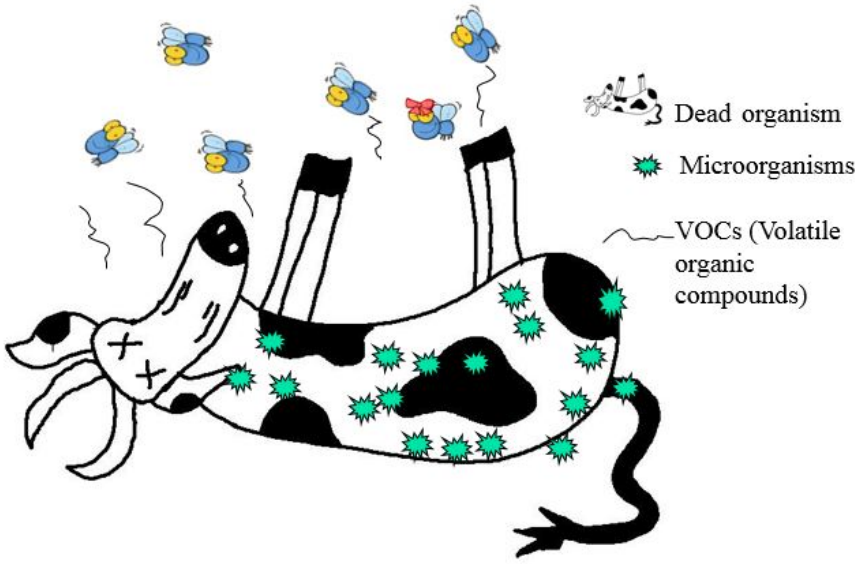


Figure 1. Carrion associated VOCs released through microbial degradation regulate fly olfactory responses.

Attraction and colonization of sheep wool by blow flies could be dependent on the concentration and composition of associated VOCs. Similarly, some VOCs released by decaying cadavers will attract *L. sericata* [77]. However, the concentration effect of

the VOCs on the behavioral response of the flies and the potential reason responsible for the observed behavior still remains unknown.

The VOCs produced by bacteria are known to be an important mechanism governing arthropod behavior. VOCs produced by a mixture of human skin bacteria grown on agar attract *Anopheles gambiae* Giles *sensu stricto* (Diptera: Culicidae) [78]. However, not all microbes present on human skin contribute equally to the level of mosquito attraction. In an assay using *Bacillus subtilis*, *Brevibacterium epidermidis*, *Corynebacterium minutissimum,* and *S. epidermidis,* which are associated with the human skin, showed that *C. minutissimum* was significantly more attractive than *B. subtilis* and *S. epidermidis* to *An. gambiae* [79, 80].

Microbial growth patterns influence VOCs production. Butyl 2-methylbutanoate, pentathiane and 2-pentadecanone, which were attractive to *An. gambiae* were more abundant in broths when the individual bacteria previously mentioned were in the stationary phase rather than in the exponential growth phase. As expected, the VOCs released in the stationary phase were attractive *to An. gambiae* while those associated with bacteria in the exponential growth phase were not [81].

Whether the compounds that attract mosquitos are also involved in the QS, which is the regulation of gene expression in bacteria as a function of the concentration of produced small molecules that reflect bacteria cell density [82], remains unknown. If those VOCs produced by the bacteria that are associated with attracting mosquitoes are involved in bacteria QS pathways, it is thus not surprising to expect to see the difference

of abundance of the VOCs detected in different stage of bacterial growth since the amount of QS molecules are dependent on cell numbers.

Gram-positive and Gram-negative bacteria use QS communication to regulate their behavior, including swarming, symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation and biofilm formation [83-85]. We have constructed a series of swarming deficient strains of *Proteus mirabilis* through mutagenesis. These mutants showed decreased swarming ability compared to wild type strain, and the genes related to swarming in *P. mirabilis* were identified through sequencing, including genes related to metabolism (*hybG, proC, pdxA, adhE* and *fadE*), regulation (*fis*, *PMI2857* and *yojN*), transcription/translation (*PMIr001*, *pnp, rhlB, rpsM*, *rrfG*, *ugd* and ureR), cell surface (*rfaL* and *zapB*), flagella (*flgK* and *flhD*). Furthermore, complementation of swarming mutations via known fly attractants showed that fly attractants, such as lactic acid, phenol, NaOH, KOH and ammonia, restored swarming behaviors of bacterial cells with the swarming mutations through mutagenesis [67]. Further analysis showed that wild type *Proteus* attracted more adult flies and induced higher chances of oviposition compared to mutant strains with reduced swarming motility, and the gas chromatography demonstrated four significant VOCs that are within the different emission patterns produced from the wild type and mutant *Proteus*. Indicating their roles in the bacterial cell-to cell communication as well as regulation of behaviors of adult blow fly [67, 74]. These four VOCs are DMDS, phenylethylalcohol, indole and isobutylamine, which are the most differentially regulated VOCs, indicating

their potential roles in interkingdom interactions between bacteria and *L. sericata* flies [74].

DMDS is a known bacterial VOC [86, 87], associated with human skin [88]. DMDS is confirmed as the VOC given off by the fly-attracting plant known as dead-horse arum (*Helicodiceros muscivorus*) and attracts flies typically attracted to decaying meat [86]. DMDS is attractive to the mosquito *Aedes aegypti* L. (Diptera: Culicidae) individually or in blends with lactic acid and acetone.[89-91]. A previous research showed female blow flies were attracted to DMDS [77], however the study did not take into account the physiological status of the flies in regard to their response to the VOC, and the oversimplified statistical analysis might have resulted in a naive interpretation of their results if one type of fly is more attracted to DMDS than another.

DMDS is a breakdown product from sulphur-containing amino acids such as methionine and cysteine [86, 92] as a result of microbial degradation, (e.g., *Proteus vulgaris*, *P. mirabilis*, *Proteus morganii*, *Providencia rettgeri*, *Proteus inconstans*) [86, 92]. Methionine goes through deamination, demethiolation and oxidation, and then DMDS will be formed [93]. Studies have shown that DMDS might be formed early in the exponential phase of growth and therefore could be used as an indicatorof *Proteus* spp. by head-space gas-liquid chromatography [86, 92].

Methionine is an essential amino acid, which cannot be synthesized de novo by insects and is most likely produced by a bacterial symbiont in many cases. The pathway of biosynthesis of methionine has been studied in *Salmomella typhimuriu*, *P. mirabilis* and *P. aeruginosa* [94-96]. Methionine enhance swarming motility of *Proteus* strains on

M9 minimal medium with significant increased protease activity (33.7–48.3 units/ml), and cellular RNA concentration [95, 97]. It can also increase *Drosophila*'s fecundity without reducing its lifespan [98]. Orthologous gene clusters were identified as shared among *Providencia* strains of *Providencia sneebia, P. rettgeri, Providencia alcalifaciens, and Providencia burhodogranariea* isolated from *D. melanogaster*, among which the gene encoding homocysteine methyltransferase was identified and the methionine biosynthesis system was designated through bacterial genome annotation [99].

Two antibacterial compounds produced by a strain of *P. mirabilis* isolated from screwworm larvae were identified as phenylacetic acid and phenylacetaldehyde, which are also widely found in fruit and other plant tissues that serve as food sources and oviposition sites for *Drosophila*. Phenylacetic acid (PAA) is produced by many bacteria as an antifungal agent [100]. It is a catabolite of the essential amino acid phenylalanine and it is also the oxidation product of phenethylamine. Phenethylamine can also be biosynthesized from phenylalanine though decarboxylation. The ubiquity of phenylacetic acid in vegetal tissues may be linked with its production by plant-associated microorganisms [100]. Besides the characteristics of antibiotic agent and its association with the common bacteria *Proteus* isolated from *L. sericata*, phenylacetic acid also has similarity in structure as well as sharing the same decomposing pathway with phenylethyl alcohol [101].

Indole is an aromatic heterocyclic organic compound. A five-membered nitrogen-containing pyrrole ring make up the bicyclic structure in indole [102]. Indole has been used as a diagnostic marker for the identification of *E. coli* [103].

It is formed from tryptophan, which is an essential amino acid that used by body to synthesize the proteins it needs by tryptophanase enzyme, encoded by the *tna* gene [104]. Indole acts as an extracellular signaling molecule and activate the *astD, tnaB,* and *gabT* genes in a concentration-dependent manner [105], and inhibits biofilm formation of enterohemorrhagic *E. coli* O157:H7 (EHEC) [106]. It is also a well-known fly attractant [107], combined with other compounds (eg., hydrogen sulphid, ethyl mercaptan and ammonia carbonate) all attracted and induced oviposition of *L. sericata* only when placed on sheep, but showed no activity when used alone [108-110]. However, no study has been carried out yet to observe the indole dose response of *L. sericata* of their life history traits and physiological state.

Isobutylamine is formed through decarboxylation by valine [111]. The reaction of the amine has been studied in a wide range of bacteria including *Proteus* spp. [112-115]. Proom and Woiwod [114] found that paper chromatograms of the culture filtrates of *Proteus* spp. mixed with casein hydrolysate showed two ninhydrin-positive substances, of which were identified as iso- butyl- and isoamylamines, arising from valine and leucine, respectively. It was further confirmed that *Proteus vulgaris* produced valine through decarboxylation [115].

Importance of microbes in insect nutrition

Microbes acquired either horizontally (e.g., from the growing development) or vertically (from mother to offspring) can play a crucial role in insect nutritional ecology by aiding in digestion of food or supplying nutrients, such as essential amino acids, that

are limited or lacking in the diet due to their limited biosynthetic capabilities [116-120]. One obligate endosymbiont, *Buchnera aphidicola* has been shown to contribute directly to aphid fitness [121] by producing essential amino acids that the aphids cannot receive from the plant sap. In addition, genome sequence also confirmed the contribution of essential amino acids synthesis by the symbiont to its host insect [122]. *Blattabacterium* strains isolated from cockroach, *Blattella germanica*, (Linnaeus) (Blattodea: Blattellidae) are involved in providing essential amino acids to the host insect [123]. In *Glossina morsitans* (Wiedemann) (Diptera: Glossinidae), its endosymbionts *Wigglesworthia glossinidia* was proved to synthesize essential vitamins to flies that cannot be obtained from vertebrate blood meal [124].

The growth of insects on sterile diets were compared to growth on rich diets to investigate the potential role of microbes in insect nutrition. The larvae of *Dacus jarvisi* (Tryon) (Diptera: Tephritidae) failed to develop on a sterile artificial medium but developed normally on the medium containing *Enterobacter cloacae*, which had been isolated from the flies [125]. The mixed cultures of *Acinetobacter* sp., *Bacillus pumilus*, *Pseudomonas mendocina*, resulted in higher average pupal weight of the horn fly, *Haematobia irritans* (Linnaeus) (Diptera: Muscidae), compared to pure cultures [126].

Bioassays demonstrated that the sand fly, *Lutzomyia longipalpis* (Lutz & Neiva) (Diptera: Psychodidae) larvae can develop on sterile rabbit feces, but the development time to adult stage was extended 12 days and survival of larvae was significantly lower (77.8 % vs 91.7 %) compared to larvae developing in the control rabbit feces with bacteria present. Furthermore, different bacteria isolated from the development

environment contributed to larval development to various degrees; for example. *Rhizobium radiobacter* supported larval development to adult stage into the greatest extent (39 days, 88.0%) while *Bacillus* spp. extended development time while reduced emergence rate (76 days, 36.0 %) [119]. Bacteria of *Ochrobactrum sp., Enterococcus faecalis, Providencia* sp. and *E. coli* O157:H7 were isolated and identified from the digestive tract of screwworm fly, *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae), and it showed that *Ochrobactrum* sp. and *E. faecalis* aided in larval development [127].

In carrion decomposition, microbes can liberate nutrients such as essential amino acids from carrion resource by digesting the tissues [128]. *L. sericata* larvae grow and develop on the carrion can consume the nutrients liberated by microbes present on the carrion [128]. In addition, commensal bacteria *P. mirabilis* has been recurrently isolated of *L. sericata* larvae [67]. This bacterium can survive the complex anti-bacterial defense system of this insect [41], which might indicate a symbiotic relationship [41]. Although the nutritional interactions between this commensal bacteria and *L. sericata* still remains to be elucidated, the pathway of biosynthesis of methionine, an essential amino acid has been studied and identified in *P. mirabilis* [95], also, the methionine biosynthesis system was designated through bacterial genome annotation of some *Providencia* strains, namely *Providencia sneebia, Providencia rettgeri, Providencia alcalifaciens,* and *Providencia burhodogranariea* isolated from *D. melanogaster* [99]. This same relationship has been discovered for *Providencia* strains, namely *Providencia sneebia,*

Providencia rettgeri, *Providencia alcalifaciens*, and *Providencia burhodogranariea* isolated from *Drosophila melanogaster* (Meigen) (Diptera*:* Drosophilidae) [99].

Importance of VOCs for attraction and oviposition by blow flies

VOCs released from decomposing materials, such as carrion, attract a wide range of insects including blow flies [129-132]. Attractant cues help blow flies locate where potential food sources, mates and oviposition sites are. At some concentrations, these same compounds could potentially serve as repellents in association with predators [67, 133]. Blow fly traps are used to reduce fly density and strike incidence by taking advantage of attraction to VOCs [107]. Studies have focused on identification of volatile components emitted under natural conditions that attract sheep blowflies [107, 130]. Ethyl mercaptan in combination with ammonia carbonate attracted blow fly *L. sericata* only when was placed on sheep. It was then furthered hypothesized that the it was the sulphur compound that resulted from the breakdown of sulphur containing amino acids, such as cysteine and methionine that contained in the fleece keratin rendered the sheep to be attracted by blow flies [134, 135]. The field study showed that the attraction depended on two factors; one was supplied by sheep, which was indicated as factor S, and the other supplied by putrefying material or certain products of putrefaction, such as ammonium carbonate, indole or skatole, which were indicted in whole as the P factor [109].

It is difficult to draw a clear distinction between attractions that lead insects to oviposition sites and those to their food [133]. VOCs with odorous emanations mediate

host-seeking behavior in mosquitoes *An. gambiae* Giles *sensu stricto* (Diptera: Culicidae) to feed on humans [81]. However, their oviposition site are quite distinct from where they are directed to for food [136]. Stimuli responsible or causing attraction does not necessarily result in oviposition by blow flies [109, 110]. An oviposition response consists of two distinct phases, attraction and oviposition [108, 109]. The first one is attraction from a distance, which in the case of attraction of sheep it depends on S factor (supplies of sheep) and P factor (products of protein decomposition). The first depends upon two factors in the case of sheep, supplied by the live animal and by products of protein decomposition [108, 109]. Similarly, the attraction of flies may due to the presence of putrefactive products and other substances. The second phase, oviposition, was initiated by attraction of flies to certain chemicals, however a meal was indicated to stimulate oviposition, by which the abdomen will be distended and will be more readily to make contact with the object [130]. Distinctions between attraction and oviposition are sometimes dictated by the physiological state of a fly. For instance, once a mosquito has had a blood meal, her physiological state shifts in numerous ways. Such shifts can result in changes in behavioral responses to chemical odors [137] .

Failure to take into account the varying concentrations of the VOCs under test led to divergent conclusions reached by different workers about the chemotropisms of the house fly [138-140]. The effect of a given compound vary with its concentration, and an initial attraction may change to strong repellence at a higher concentration [138]. The effects of carbon dioxide and of ammonia as attractant and stimuli for oviposition by the housefly, *Musca domestica L,* were in dispute between Richardson [139] and Crumb &

Lyon [140]. Ammonia was indicated by Richardson to be the primary compound found in decomposition material to stimulate oviposition [139] whereas Crumb and Lyon found that carbon dioxide was the major attraction for egg deposition and they indicated that ammonia was actually repellent [140]. However, the concentration of any volatile compounds studied in those research remains unknown. Behavioral responses of an arthropod can be dose specific. For example, the response of *A. aegypti* to DEET (N,N-diethyl-m-toluamide) was dose specific with attraction observed when the compound was at low concentrations [141]. For DMDS, when at the lowest concentration and blended with ammonia, lactic acid, and tetradecanoic acid, *An. gambiae* attraction greater than compared to the pure compound at higher concentrations or basic blend alone [81]. Ammonia, a protein metabolite produced by bacteria [142, 143] attracted house fly, *Musca domestica* L. (Diptera: Muscidae) over a limited range of low concentration but was strongly repellent at higher concentrations [138].

Physiological status and sex of an insect influences its response to VOCs [74]. Gravid *L. sericata* showed significant different responses to *Proteus* than non-gravid flies [144]. In fruit flies, males of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) are more responsive to kerosene, which was used as an attractant for male flies in Australia [145]. Female house flies, *Musca domestica* L., (Diptera: Muscidae) are more strongly attracted than males to ammonia and vice versa with respect to ethyl alcohol [138]. For *D. melanogaster*, adult females respond strongly to ethyl alcohol up to 25%, with a maximum response at 10-15%; and *Drosophila* males exhibited a maximum response at or below 5% and to ethyl alcohol up to 15% [146]. *Drosophila* females were

also positive to acetic acid in concentrations up to 1%, with a maximum response at 0.4% while males are positive to acetic acid in concentrations up to 1%, with a maximum at 0.2% [146]. Hoskins and Craig, by showing the quantitative response of *L. sericata* to different concentrations of select VOCs, demonstrated that attraction to ammonia at a lower concentration (0.012%) was attractive but repellent at concentrations greater than 0.03% [147]. No response of flies to carbon dioxide was showed until the concentration adds up to 2% [147]. Different sexes of *L. sericata* responded differently to the target with females more strongly attracted than males to ammonia while males are more strongly attracted to ethyl alcohol [147]. Hobson pointed out that non-gravid blow flies were attracted to carrion but migrated to sheep when they were gravid to oviposit, indicating the factors presence on sheep were specific for gravid females [81].

Nutritional status affects fly responses as well. Tomberlin et al. [74] determined that fewer milk-fed females were gravid compared with those fed blood from beef liver, and sex and ovarian state had significant effect on choice of wild type *Proteus* exhibiting QS response versus a mutant [74]. Age also affects the response of flies to resources [74]. Younger flies exhibited a clearer choice while older flies were more variable [74, 148].

The responses of flies to the VOCs emitted from a resource, such as carrion, are affected by physiological status, sex classes [74] as well as the different doses of the VOCs [138-140]. Bacteria play an important role in release the VOCs from the resource [149]. These VOCs might be involved in bacterial QS communication as well as regulation of flies' behaviors in response to the resource such as whether to consume the

food, to oviposit, to start the mating [74], or simply just fly away (eg. if the VOCs are repellent to them) etc. [133]. On the other side, the exposure to VOCs not just enact the behaviors of insects [74], it may in turn also impact the physiology of insects [150-152].

Impact of VOCs on life-history traits of arthropods

Detection of VOCs impacts adult physiology and aging in *Drosophila*, through the perception of nutritional resources. The olfaction system allows many arthropods, such as blow flies, to locate resources vital for their survival. Exposure to nutrient derived odorants, e.g. yeast odorant [150-152], can modulate life span and partially reverse the longevity-extending effects of dietary restriction [150]. Exposure to yeast odorants reduced the life span of *Drosophila* subjected to dietary restrictions, while fully fed flies were not impacted [150]. Diet- and odorant-mediated regulation of aging may act at partly through the same molecular pathway [150]. Mutation of odorant receptor Or83b resulted in severe olfactory defects, altered adult metabolism, enhanced stress resistance, and extended life span [150]. Another example is carbon dioxide, which is an important component of complex, food based smells and provides information about nutrient availability, and impacts the response of flies to profitable food sources [153]. The population of olfactory neurons that express receptor Gr63a, which is a component of the olfactory receptor for gaseous phase carbon dioxide, is required for yeast odorant to regulate fly life span. The yeast odor failed to impact the life span on flies with deletion in Gr63a function. These results suggest that carbon dioxide plays a role in the detection of live yeast and regulation of fly life span with perceived nutrition availability

[150, 154]. *D. melanogaster* exhibits an innate olfactory-based avoidance behavior to carbon dioxide, a component of odor emitted from stressed flies[155] while certain food sources that emit carbon dioxide also emit odorants that reduce carbon dioxide sensitivity and then inhibit avoidance behaviors of *D. melanogaster* [156]. PAA is widely found in fruit and other plant tissues that serve as food sources and oviposition sites for *Drosophilia* [157]. Phenylacetic acid and phenylacetaldehyde were detected in whole-body cuticular extracts *of D. melanogaster* [144]. The activation of ionotropic receptor 84a (IR84a) neuron, which is a member of the chemosensory ionotropic glutamate receptor family by odors of phenylacetic acid and phenylacetaldehyde, can promotes male courtship in *Drosophila*, indicating the role of these compounds in the biology of *Drosophila* [144].

DMDS, indole, phenylethyl alcohol and Isobutylamine were indicated as potential bacterial QS molecules and regulate fly attraction and oviposition to carrion associated resource [67, 74]. PAA has similarity in structure as well as sharing the same decomposing pathway with Phenylethyl alcohol [101], also it can be by the common bacteria *Proteus* isolated from *L. sericata*, and as designated as “mirabilicids“ due to its antimicrobial activity [53].

Objectives

Recent studies indicated potential interkingdom VOCs including DMDS, indole, phenylethyl alcohol and isobutylamine in regulations of bacterial cell-to-cell signaling systems as well behaviors of blow flies to potential resources. Previous research

documented the attractiveness of indole [108-110] and DMDS [77] to flies**.** However, there are still questions remain to be clarified, which are the main experiment questions in my research (Figure 2), including 1, the effect of physiological status of blow flies, such as sex and ovarian development, in their response to these VOCs. 2, the physiological effects of detection of these VOCs on life history related traits of blow flies. And 3, effects on the larval preference and performance on amino acid deficient diets.

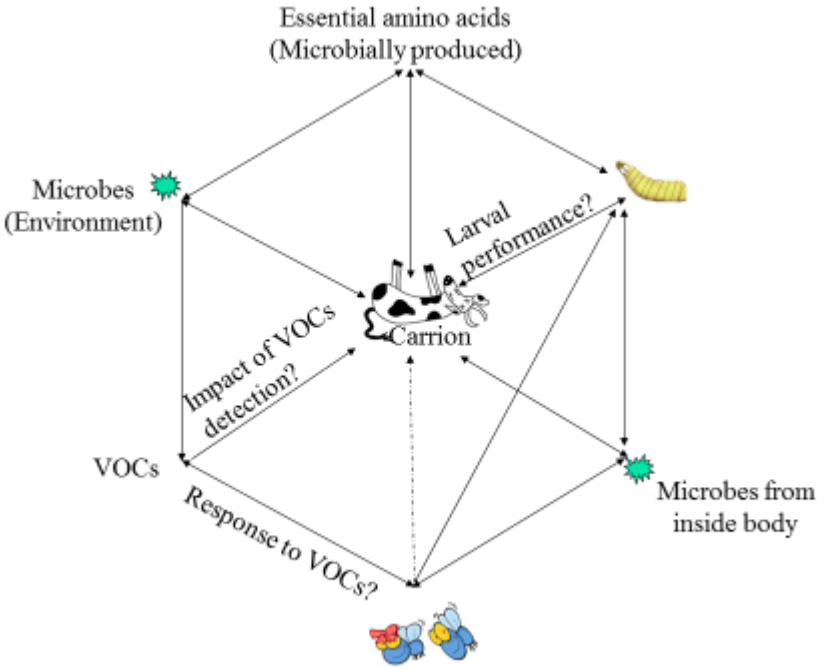


Figure 2. Key questions in dissertation. Question1: How do flies respond to these associated VOCs? Question 2: How do larvae perform on carrion associated resources as a result of both maternal decision

and their own carrion utilization strategies? Questions 3: What is the impact of detection of VOCs on fly physiology?

The goals of my research include:

1. Construct and confirm chromosomal marked *gfp Providencia* sp. Previous studies demonstrated some aspects of the interkingdom relationship between *L. sericata* and *P. mirabilis* which was originally co-isolated with *Providencia* from *L. sericata* salivary glands [67]. It demonstrated that swarming deficient *P. mirabilis*, mutants that are deficient in QS signaling pathways, results in reduced *L. sericata* attraction and oviposition [74]. Based on the preliminary data, I propose that interkingdom interactions may also exist between *L. sericata* and the associated *Providencia* spp. I thus constructed the *gfp*-tagged *Providencia* as tools for investigate of the interaction of *Providencia* species with *L. sericata* in order to study the fate of the bacteria after being ingested by *L. sericata* and how the physiology of the flies will be affected by the bacteria. GFP producing *Providencia* would be used to visualize bacteria along the alimentary canal of *L. sericata* larvae. Also, it is difficult to tell apart *Providencia* and *Proteus* based on phenotypes showed on agar culture, *gfp*-tagged bacteria will eliminate the problem. Also, the future test of viability/quantification of *Providencia* inside after ingestion by *L. sericata* larvae will involve the dissection and separation of the gut sections, the *gfp*-tagged bacteria will reduce confusing them with other bacteria already present or introduced into the study system through the experimental processes.
2. The investigation of response of *L. sericata* to VOCs that associated with

carrion/ larval resources, including how the dose effect of different volatile compounds and different physiological states and sex of flies affect their responses. DMDS, indole, phenylethyl alcohol and isobutylamine were indicated as potential bacterial QS molecules and regulate fly attraction and oviposition to carrion associated resource [67, 74]. PAA has similarity in structure as well as sharing the same decomposing pathway with Phenylethyl alcohol [101], also it can be by the common bacteria *Proteus* isolated from *L. sericata*, and as designated as “mirabilicids“ due to its antimicrobial activity [53]. Thus, in my study I decided to use DMDS, indole, PAA and isobutylamine to study the response of *L. sericata* to carrion associated VOCs.

Flies of different biological states may have different ecological roles, I thus hypothesize that the responsiveness of blow fly to these VOCs may be different based on the different doses of the compounds as well as the physiological status and sex of the flies.

3. Studying larval performance in terms of their food preferences and development on imbalanced artificial amino acid diets. Previous work determined four compounds that are potential chemicals in regulation of the attraction and oviposition of adult *L. sericata* [74]. These chemicals, DMDS, isobutylamine, indole, phenyacetic acid, can be produced through microbial decomposition, indicating presence or absence of carrion associated resource. Adult blow flies were indicated to use MVOCs as cues to exploit the resources such as food, host, appropriate oviposition sites [158]. Attraction and colonization of carrion

resource by adult flies are followed by the subsequent development of offspring. However, little is known about larval performance on carrion associated resource as the consequences of both female oviposition site selection and their strategies on resource utilization. Just like adult blow flies, larvae need carrion resource for survival as well [149]. However, unlike adult flies, larvae have very limited mobility once hatched out from eggs, thus, on one side, larval performance is largely affected by adult flies decisions of resource selection, on the other side, it is critical for them to develop strategies in resource utilization considering the fact that carrion is an ephemeral resource [149] and heterogeneous in terms of nutrition and distribution nutrition [159]. Insects prefer to lay their eggs on microbe rich environments compare to sterile environments [128, 160]. In addition, microbes may also contribute to larval development by liberating essential amino acids from carrion resource by digesting the tissues for larval consumption [128] or directly supplying essential amino acids that are limited or lacking in the diet due to their limited biosynthetic capabilities [116-118]. Also, wild type *Proteus* induced higher *L. sericata* attraction and oviposition compared to mutant *Proteus*, and the emission patterns of VOCs from these bacteria differed [74]. DMDS, indole, PAA and isobutylamine are breakdown products of essential amino acids of methionine, tryptophan and phenylalanine and valine individually. In order to better how mom's choose the carrion resource and the effect of the resource on the larval performance (the association between adult behaviors and the subsequent larval performance), a research was carried

out to analyze food preference and development of larvae on imbalanced essential amino acids dietary deficient in one of the four essential amino acids as mentioned above. Besides, antimicrobials were added into the diet to study the potential roles of microbes on larvae performance. I hypothesized that the imbalance in dietary amino acids and the presence of microbes will affect larval food preference and development.

4. The effect of VOCs on life history related traits of *L. sericata*. DMDS has been previously identified as potential bacterial QS molecules and its potential roles in the regulation of files' attraction and ovipostion to carrion associated resource was indicated [67, 74], however its effect on the life history related traits of *L. sericata* remains unknown. Based on previous research that exposure to resource associated odor affected life span related traits of insects [150-152, 154]. I hypothesized that the effects of exposure to different DMDS concentrations on history related traits of *L. sericata* will be different based on their sex and age range. Investigation will involve the life span and number of defecation spots of different age groups and sex of *L. sericata* after exposure to different DMDS concentrations. While life span has been immensely used to study the environment and genetic effects [150], insect defecation is relatively less studied as related to life-history traits in response to perception of carrion associated VOCs.

CHAPTER II

CONSTRUCTION AND CONFIRMATION OF GFP-TAGGED *PROVIDENCIA* SP. STRAINS

Introduction

The green blow fly, *L. sericata* is a cosmopolitan species of great medical, veterinary and forensic importance [1, 2]. *L. sericata* is an important vector of many human and animal pathogens [17]. Adults and larvae carry pathogens in their body after consuming material from contaminated sources, such as animal carcasses and feces [18, 19]. However, commensal bacteria have also been determined to contribute to the success of maggot treatment. For example, *Proteus mirabilis* is considered a commensal in the larval gut of *L. sericata*, and it produces agents that are lethal to Gram-positive and Gram-negative bacteria [19]. The agents produced by *P. mirabilis* that kill other bacteria are referred to as ‘‘mirabilicides’’. These compounds have been determined to kill microorganisms, such as *Bacillus subtilis* and *Candida albicans*, present in food material or wounds [19]. Additionally, *P. mirabilis* is able to shift the pH of a wound which suppresses other bacteria, such as *S. aureus* [19]. Two “mirabilicides” produced by *P. mirabilis* in the gut of screwworm larvae, *Cochliomyia hominovorax* (Diptera: Calliphoridae) are phenylacetic acid and phenylacetaldehyde to be produced by *P. mirabilis* [161].

Providencia spp. are Gram negative bacteria [162], and have been recurrently co-isolated with other bacteria from *L. sericata* salivary gland [67]. Historically, *Proteus*

and *Providencia* were placed in a same tribe called *Proteeae*, since they have similar phenotypic traits such as positive for phenylalanine deaminase and negative for arginine decarboxylase [163]. Besides, *Providencia* and *Proteus* are both responsible for the formation of crystalline bacterial biofilms on urethral catheters, that are resistant to antibiotics [70]. One of the most common *Providencia* sp., *Providencia stuartii*, is an opportunistic pathogen of humans and can cause urinary tract infections with urinary catheter [68]. The urease produced by this bacterium plays important roles in kidney and bladder stone formation [164].

The association of *Providencia* spp. with *L. sericata* is still understudied. The communication between *P. stuartii* with flies was previously demonstrated by the inner membrane protein AarA encoded by *P. stuartii* that is required for the release of an extracellular quorum-sensing signal shared with fruit flies (Diptera: Drosophilidae) [73]. This study demonstrated that the expression of *P. stuartii aarA* rescued wing vein development in a *D. melanogaster rho* mutant, whereas expression of fly *rho* in a *P. stuartii aarA* mutant rescued the cell communication phenotype associated with *aarA* mutation. It was based on the fact that the protein AarA is homology to RHO and they could rescue the mutant phenotypes of each other when exogenetically expressed, that it concluded that there would be potential cell to cell communication pathways shared between this bacteria and the insect.

Providencia spp. have been recurrently co-isolated with other bacteria from *L. sericata* salivary gland [67]. Because of the association between *L. sericata* and *Providencia* spp., it is possible they could serve as a new model system for studying

general host-pathogen interactions, and for understanding how *L. sericata* interacts with its commensally bacteria. Previous studies demonstrated some aspects of the interkingdom relationship between *L. sericata* and *P. mirabilis* which was originally isolated from *L. sericata* salivary glands [67]. The potential interkingdom relationship between *Providencia* spp., which were recurrently co-isolated with *P. mirabilis*, and *L. sericata* remains to be elucidated.

It has been documented that the reduction of *E. coli* varied across different sections of the *L. sericata* gut with bacteria concentration decreased significantly more in the anterior hindgut than what was observed in the fore or midgut; no bacteria were seen in the posterior end near the anus [165]. In order to investigate the fate of *Providencia* spp. after ingestion, green florescence protein (GFP) producing *Providencia* would be used to visualize bacteria along the alimentary canal of *L. sericata* larvae. The future evaluation of internal viability/quantification of *Providencia* after ingestion by *L. sericata* larvae will involve the dissection and separation of the gut sections, the *gfp*-tagged bacteria will reduce confusing them with other bacteria already present or introduced into the study system through the experimental processes.

The construction of *gfp*-tagged *Providencia* strains can be achieved through bi-parental mating by *Providencia* spp. with *E. coli* pUT*gfp*2 (S17-1 λ*pir*). The bacterial plasmid in *E. coli* pUT*gfp*2 (S17-1 λ*pir*) carries kanamycin resistance. The plasmid transposon will transfer kanamycin resistance gene and insert *gfp* to recipient strains' chromosome after mating. If successful, the *gfp*-tagged *Providencia* spp. will glow similarly as the donor strain of *E. coli* pUT*gfp*2 (S17-1 λ*pir*). Consequently, it would not

be possible to separate the two on Luria-Bertani (LB) plates. Therefore, it is necessary to carry out a confirmation of these constructed *gfp*-tagged strains to exclude *E. coli* or any other potential bacterial contaminations.

Chromogenic agar is a differential culture medium that facilitates the rapid recovery and identification of specific microbial species or the isolation of bacteria that can be identified at the genus level [166]. The pigmentation of the growing colony on the agar media was one of the first important observable clue in the process of differentiating and identifying bacterial colonies [167]. However, colony color expressed in the conventional medium without selective pressure can be misleading, as different genera of microorganisms can have similar color hues which makes it difficult for identification, for example *Micrococcus luteus* and *S. aureus* all express pale yellow hues, the *Pseudomonas* spp. express various shades of green and red. The use of chromogenic medium is a better way than conventional microbiological agar media for growth, isolation and identification of bacteria [166]. Chromogenic agar is both selective and identifying [168], the principles of which relies on the use of selective media with a proprietary carbohydrate substrate- complex that is enzymatically hydrolyzed by the targeted bacteria, and results in specific pigmented colony due to the residual chromogen accumulation in the bacterial cell. The colonies of *E. coli* pUT*gfp*2 (S17-1 *λpir*) and the constructed *gfp*-tagged *Providencia* spp. display of similar color in conventional agar medium and similar green fluorescence under the ultra violet microscope, the distinctions of which were confounding, therefore chromogenic agar will aid in distinguishing between the donor and recipient strains, though it cannot provide recipient

identity. One of the commercially used chromogenic agar, HardyCHROM™ Coliform EC (Hardy Diagnostics, Santa Maria, CA) [169], can be used for detection, differentiation, and enumeration of *E. coli* and other coliforms based on colony color. *E. coli* can be identified as pink to violet colored colonies on the plate, while other coliform bacteria will appear as turquoise colonies. However, there are no commercially available Chromogenic cultures for positive identification *Providencia* spp. based on positive results. Thus, by using the HardyCHROM™ Coliform ECC medium, is only able to specifically separate *E. coli* from other coliform bacteria based on the specific colonial colors [170], while does not directly allow identification for *Providencia* spp. [170]. In order to have a more specific identification, restriction enzyme were used in this research to differentiate *gfp*-tagged *Providencia* spp. from the *E. coli* pUT*gfp*2 (S17-1 λ*pir*) besides the use of Chromogenic medium.

Restriction enzyme digestion of bacterial DNA provides a discriminatory method of bacterial characterization [171]. Restriction enzymes (or restriction endonuclease) recognize and cleave DNA at specific recognition sites on the sequences [172-174]. The resulting fragments can be separated by gel electrophoresis to give a characteristic banding pattern. The different digestion patterns enables differentiation of bacterial species [171]. The choice of restriction enzyme for a particular bacterial species is critical; a preferable enzyme is the one that results in distinct pattern with discernible number of discrete bands, and most important, allows differentiation of the bacteria species/strains to be distinguish. Since the complete sequences of the 16S rRNA (ribosomal RNA) gene of these bacteria used in the research were unknown (except only

the partial 16S rRNA gene sequence of the reference *Providencia* spp.), the reference sequences from NCBI database were therefore used to design primer pairs and choose enzymes. Newly designed primer pair were supposed to amplify the 16S rRNA gene of *E. coli* and *Providencia* used in the research, and then restriction enzymes, PflFI and ApoI, that have different cutter frequency and cutting locations in the tested polymerase chain reaction (PCR) products resulted in different restriction enzyme patterns of *E. coli* and *gfp* –tagged *Providencia and* wild type *Providencia* sp. The combination of restriction patterns thus allowed for more specific validation of these *gfp*-tagged *Providencia* strains. The objectives of the research in this part were to construct the *gfp*-tagged *Providencia* strains from a wild type *Providencia* sp. that had been isolated from *L. sericata* salivary glands, and to confirm those *gfp*-tagged strains after *gfp* construction. The confirmed *gfp*-tagged *Providencia* strains would be used to study *Providencia* spp. in the gut of *L. sericata*.

Methods

Construction of chromosomal marked (gfp marked) Providencia sp.

A wild type *Providencia* sp. was isolated from *L. sericata* salivary glands, and were used as recipient strains. *E. coli* pUT*gfp*2 (S17-1 λ*pir*) was used as donor strain providing *gfp* sequence and chromosomally transfer into target cells. Filter system was used for bi-parental mating of the recipient and donor cells [175].

The bacterial plasmid in *E. coli* pUT*gfp*2 (S17-1 λ*pir*) carries kanamycin resistance. The plasmid transposon will transfer kanamycin resistance gene and insert

gfp to recipient strains' chromosome after mating. Rifampacin resistance was induced to *Providencia* sp. as selective marker. Antibiotic construction for recipient strains was by serial growth into gradually increased concentrations of rifampacin in LB broth. *Providencia* sp. would finally be transferred to LB medium supplemented with rifampicin in 64 μg/ml. Exconjugants were selected in agar plates with both antibiotics: Kanamycin (50 μg/mL) and rifampacin (64 μg/mL).

Approximately 29.6g of LB medium (Miller BL7213, NEOGEN Lansing, MI, USA) was dissolved in 800mL purified RO water in heat using Hotplate Magnetic Stirrer (Benchmark Scientific, Inc, South Plainfield, NJ, USA), and then autoclaved (STAGE 3 autoclave, USDA Property) at 121ºC for 15 min. Kanamycin (FISHER Bioreagents, PA, USA) and Rifampacin (Sigma-Aldrich Co. LLC., USA) were added to cooled sterilized medium before poured into plates. 25 g of the LB broth medium (MILLER, NEOGEN Lansing, MI, USA) was dissolved in one liter of purified water, mixed thoroughly and then Autoclaved at 121°C for 15 minutes, sterilized LB broth was distributed into 15mL centrifuge tubes with flat top cap (CHASMA Scientific, INC.). Rifampacin (Sigma-Aldrich Co. LLC., USA) was added to tubes to make serial concentration of Rifampicin of 0.25, 0.5,1,2,4,8,16,32,64 (μg/ml) for induction antibiotic resistance in recipient *Providencia* cells for selection marker. Each 20mL of sterilized LB agar medium was poured into 100*15mm BD Falcon petri dish (BD Falcon™, Becton, Dickinson and Company, NJ, USA). LB Agar plates with 50 μg/ml kanamycin were used for *E. coli* pUT*gfp*2 (S17-1 λ*pir*) with kanamycin resistance; LB Agar plates with serial concentration of Rifampicin of 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64 (μg/ml) were

used for transferring induced Rifampacin resistant bacteria from LB broth into agar plates. LB Agar plates contained with both 50 μg/ml of kanamycin and 64 μg/ml of Rifampicin were used for exconjugants selection.

Conjugal mating of the recipient and donor cells was carried out through bi-parental mating, in which the *E. coli gfp*2 (S17-1 λ-*pir*) strain, serves as donor strain for transferring the *gfp* gene to recipient *Providencia* sp. We used filter system for bi-parental mating [175]. *Providencia* sp. with Rifampicin resistance and *E. coli pUTgfp2* (S17-1 λ*pir*) with kanamycin resistance would be retrieved from glycerol preserved in -80ºC, and then used 10 μL sterile loop (Biologix Research, KS, USA) to inoculate in the prepared LB agar plates (Miller BL7213, Neogen Lansing, MI, USA) for 18 hours at 37 ºC, and then adjusted solution with PBS till OD_{600}=0.7. A 50 μL mixture of equal volumes of both recipient strain and donor strain in PBS solution, both in stationary phase, were mixed into 5mL LB medium of 10mM $MgSO_4$, and vortexed (Vortex-Genie 2, Scientific Industries, Inc., New York, USA) a few seconds and transfer to a 5ml disposable syringe (Thermo Fisher Scientific, Waltham, MA), and filter through the sterile 0.45 μm millipore membrane (Thermo Fisher Scientific, Waltham, MA) to make retention of the bacteria. Filter membranes with the mixture of recipient and donor strains were placed on top of LB agar medium without antibiotic for 18-24 hour at 37 ºC. Bacteria were then washed from the filter with 1mL LB medium and the washed solutions were plated with LB agar plates with both 50 μg/ml kanamycin and 64 μg/ml Rifampicin for selection of *Providencia* sp. exconjugants.

Confirmation of gfp-tagged bacteria

HardyCHROM™ ECC plates (Hardy Diagnostics, Santa Maria, CA) were used for initial confirmation of *gfp*-tagged *Providencia* sp. [170]. HardyCHROM™ ECC plates were warmed to room temperature, and the agar surface were made sure was dry prior to inoculating. *gfp*-tagged *Providencia* strains were retrieved from glycerol preserved in -80ºC Ultra-Low Temperature Freezer (SANYO North America Corporation), and then grow in the LB agar plate (with 64 μg/ml Rifampicin) for 18-24 hours at 37ºC, and then used 10 μL inoculating loop (Biologix Research, Kansas, USA) to streak bacterial colonies into HardyCHROM™ ECC plates. Plates were incubated at 37ºC for 18-24 hours. Colonial morphology and color were examed. *E. coli* can be identified as pink to violet colored colonies on the plate, while other coliform bacteria will appear as turquoise colonies; other gram-negative bacteria appear as white or colorless colonies [169].

Confirmation of *gfp*- tagged *Providencia* strains by enzyme restriction patterns was carried out by using newly designed universal primers to amply the complete 16S ribosomal RNA (rRNA) gene of *Providencia* and *E. coli* pUT*gfp*2 (S17-1 λ*pir*). Primer sequences are as follows; left primer, 5'-3', ATCATGGCTCAGATTGAACG, right primer 5'-3', GCAGGTTCCCCTACGGTTA. The primers amply roughly1, 500 bp spanning the entire 16 S rRNA genes of *E. coli* pUT*gfp*2 (S17-1 λ*pir*) and *gfp*- tagged *Providencia* strains.

Bacterial DNAs were extracted from *gfp*-tagged *Providencia* strains, and *E. coli* pUT*gfp*2 (S17-1 λ*pir*) and used as templates for PCR. Bacteria were retrieved from

glycerol preserved in -80ºC Freezer (SANYO North America Corporation), and then streaked in the LB agar plates with antibiotics (as described above) for 18-24 hours at 37 ºC, and then used sterile inoculating loop 10 µL (Biologix Research, Kansas, USA) to transfer one loopful of overnight bacteria into 500 µL of DNAse/RNAse free DEPC treated ultrapure water (K.D Medical, Columbia, Maryland,USA) in 1.5 mL centrifuge tube (Fisherbrand, Fisher scientific, Waltham, MA), mixed well using vortex (Vortex-Genie 2, Scientific Industries, Inc., New York, USA) and were heated at 100ºC in multi block heater (EquipNet, Inc.Canton, MA) for 15 minutes. Tubes were then spinned down in 13,000 rpm (Fisher scientific accuspin, Thermo Fisher Scientific, Waltham, MA, USA), supernatant were collected and preserved in 4ºC refrigerators for further use.

PCR was performed to amplify the complete bacterial 16S rRNA genes for *Providencia* and *E. coli* pUT*gfp*2 (S17-1 *λpir*).Using PTC-200 thermal cycler (MJ Research, Quebec, Canada), each PCR consisted of a 50 µL reaction containing of 25uL of Qiagen Hotstar Taq master mix (QIAGEN), 1 µL of DNA template (approximately 0.1µg), 2 µL of each primer totaling 2 µM. Amplification conditions were as follows: initial denaturation at 95°C for 15 min followed by 35 cycles of denaturation at 94°C for 1min, primer annealing at 60°C for 30secs and extension at 72°C for 1 min. A final extension step was done at 72°C for 5 min.

All 16S rRNA gene PCR products were analyzed by electrophoresis on 1% agarose gel (Agarose PS1200, Biolink scientific, Wimberley, TX, USA) with Owl D2 Wide Gel separation System (Thermo Fisher Scientific, Waltham, MA, USA). 16s rRNA gene PCR products were mixed in 1:5 volume with gel loading solution (Sigma-Aldrich,

St. Louis, MO, USA), 10 μL of reaction mixture was loaded for each sample and Hi-Lo DNA ladder with a wide range of 50 to 10,000 base pairs (Minnesota Molecular Inc, MN, USA) was used to determine the size of the bands. 500-750ng of DNA products were used per well. Gels were run in 1XTAE (Tris acetic acid with EDTA) buffer (Fisher BioReagents) at 100V for 1.5 hrs. Ethidium bromide (MP biomedicals, Santa Ana, CA) with final concentration of 0.5 μg/ml in the gel enabled the DNA to be viewed using ultraviolet light.

Since the complete sequences of these PCR products were unknown, the reference sequences were used to choose enzymes, the information of which were as follows: *Providencia stuartii* MRSN 2154 chromosome, complete genome with NCBI Reference Sequence: NC_017731.1; *Providencia* sp. Sal2 16S rRNA gene, partial sequence, accession number JN790944; *E. coli* O26:H11 str. 11368 chromosome, 16S RNA gene with NCBI Reference Sequence: NC_013361.1 (complete genome); *E. coli* str. K12 substr. W3110 strain (GenBank: AP009048.1) 16S RNA gene sequence. All the restriction enzymes and the reagents used in digestion system in this research were from New England Biolabs.

The restriction enzyme PflFI (New England Biolabs, Ipswich, MA, USA) was initially used since it would only cut 16S rRNA gene sequence of *E. coli* with recognition site GACNNNGTC, according to the reference sequences. Expected sizes for resulting fragments after digestion were round 1.2kb and 0.3kb each. Before digestion, DNA concentrations of PCR products were quantified using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Restriction

enzyme incubations were set for 15 μL reactions with 9 μL *E. coli* 16S rRNA gene PCRproduct (1000ng), 1.5 μL 10×NEB Buffer#4, 1 μL PflFI (10U/uL), 1.5 μL BSA (1mg/mL) according to manufacturer's instruction, 2 μL nuclease free water (QIAGEN). Incubations were carried out in PTC-200 thermal cycler (MJ Research, Quebec, Canada) at 37℃ for overnight (16hrs) and then at 65°C for 20 minutes for enzyme inactivation. Fragments were analyzed by electrophoresis on 1% agarose gel (Agarose PS1200, Biolink scientific, Wimberley, TX, USA). 10 μL of reaction mixture with gel loading solution (Sigma-Aldrich, St. Louis, Missouri), was loaded for each sample and Hi-Lo DNA ladder with a wide range of 50 to 10,000 base pairs (Minnesota Molecular Inc, MN, USA) was used to determine the size of the bands. Electrophoresis are carried out with Owl D2 Wide Gel separation System (Thermo Fisher Scientific, Waltham, MA) at 100V for 1.5 hrs. The digested fragments of amplified 16S rRNA gene products from a wild type *Providencia* strain, whose partially 16S rRNA gene was known, was used as positive control while *E. coli* pUT*gfp*2 (S17-1 λ*pir*) *was* used as negative control.

The restriction enzyme, ApoI, was selected based on the sequence of both *Providencia* sp. Sal2 16S ribosomal RNA gene, partial sequence, accession number JN790944, and *Escherichia coli* str. K-12 strain 16S RNA gene sequence, AP009048. It has different cutters in *Providencia* and *E. coli* pUT*gfp*2 (S17-1 λ*pir*) based on the reference sequences as described above. Before digestion, DNA concentrations of PCR products were quantified using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Reaction system consists of 1.5 μL BSA (1mg/mL), DNA product 1000 ng, 10 μL ApoI, 1.5 μL NEBbuffer#3, making total volume of 15

µL. Incubations were carried out in PTC-200 thermal cycler (MJ Research, Quebec, Canada) at 50℃ for overnight (16hrs) and then at 80°C for 20 minutes for enzyme inactivation. Fragments were analyzed on 1% agarose (6Mgel Agarose, Neuvitro Corporation) gel. 10 µL of reaction mixture with gel loading solution (Sigma-Aldrich, St. Louis, Missouri) was loaded for each sample and Hi-Lo DNA ladder (Minnesota Molecular Inc, Minnesota, USA) with a wide range of 50 to 10,000 base pairs was used to determine the size of the bands. Electrophoresis was carried out with a high speed gel system. (RGX100 gel system, Neuvitro Corporation), at 220V for 20 minutes. The digested fragments of amplified 16S rRNA gene products from a wild type *Providencia* strain, whose partially 16S rRNA gene was known, was used as positive control while *E. coli* pUT*gfp*2 (S17-1 λ*pir*) *was* used as negative control.

Results

Construction of chromosomal marked (gfp marked) Providencia sp.

Through bi-parental mating of *Providencia* sp. with *E. coli* pUT*gfp*2 (S17-1 λ*pir*), 12 *gfp*-tagged strains had been constructed with name of *gfp*-tagged-(A2,A3,B1,C2,D5,D7,E7,E9,G4,H5,H6,J11).

Confirmation of gfp-tagged bacteria

After inoculation in HardyCHROM™ ECC plates and grew overnight, eleven *gfp*-tagged *Providencia*- A2, A3, B1, C2, D5, D7, E7, E9, G4, H5, H6 showed negative results with colorless colonies, which ruled out the possibility of being either *E. coli* or

Table 1. Examination of colonial color of *gfp*-tagged *Providencia* strains. -: indicates negative results, with no presence of *E. coli* or other coliform bacteria; +: indicates positive results.

Test strain name	Results	Description
Pro-gfp-A2	-	colorless colonies
Pro-gfp-A3	-	colorless colonies
Pro-gfp-B1	-	colorless colonies
Pro-gfp-C2	-	colorless colonies
Pro-gfp-D5	-	colorless colonies
Pro-gfp-D7	-	colorless colonies
Pro-gfp-E9	-	colorless colonies
Pro-gfp-G4	-	colorless colonies
Pro-gfp-H5	-	colorless colonies
Pro-gfp-H6	-	colorless colonies
Pro-gfp-J11	+	colonies with a mixed color of violet and pink

any other coliform bacteria based on the manual of interpretation of results [169]. However *gfp*-tagged Pro-J11 showed a mixed color of violet and pink (Table 1).

In order to further confirm these strains, primer pair was designed to amply the 16S rRNA gene of this *Providencia* sp., *E. coli* pUT*gfp*2 (S17-1 λ*pir*) and all the *constructed gfp-tagged* strains. Based on the complete 16 S rRNA genes of reference strains of *Providencia stuartii* MRSN 2154 (genebank: NC_017731.1) and *Escherichia coli* str. K-12 strain (genebank: AP009048), primer sequences were designed as follows Left PRIMER from 5'-3': ATCATGGCTCAGATTGAACG; right primer from 5'-3': GCAGGTTCCCCTACGGTTA, as shown in the DNA reviewer Figure 3 and Figure 4, the expected PCR products were around 1.5kb, spanning the restriction sites and

therefore allowed differentiation by enzyme restriction carried out later. Based on the partial 16S rRNA gene sequence of *Providencia* sp. Sal2, (partial sequence, accession number JN790944), its restriction patterns by ApoI are demonstrated in Figure 5.

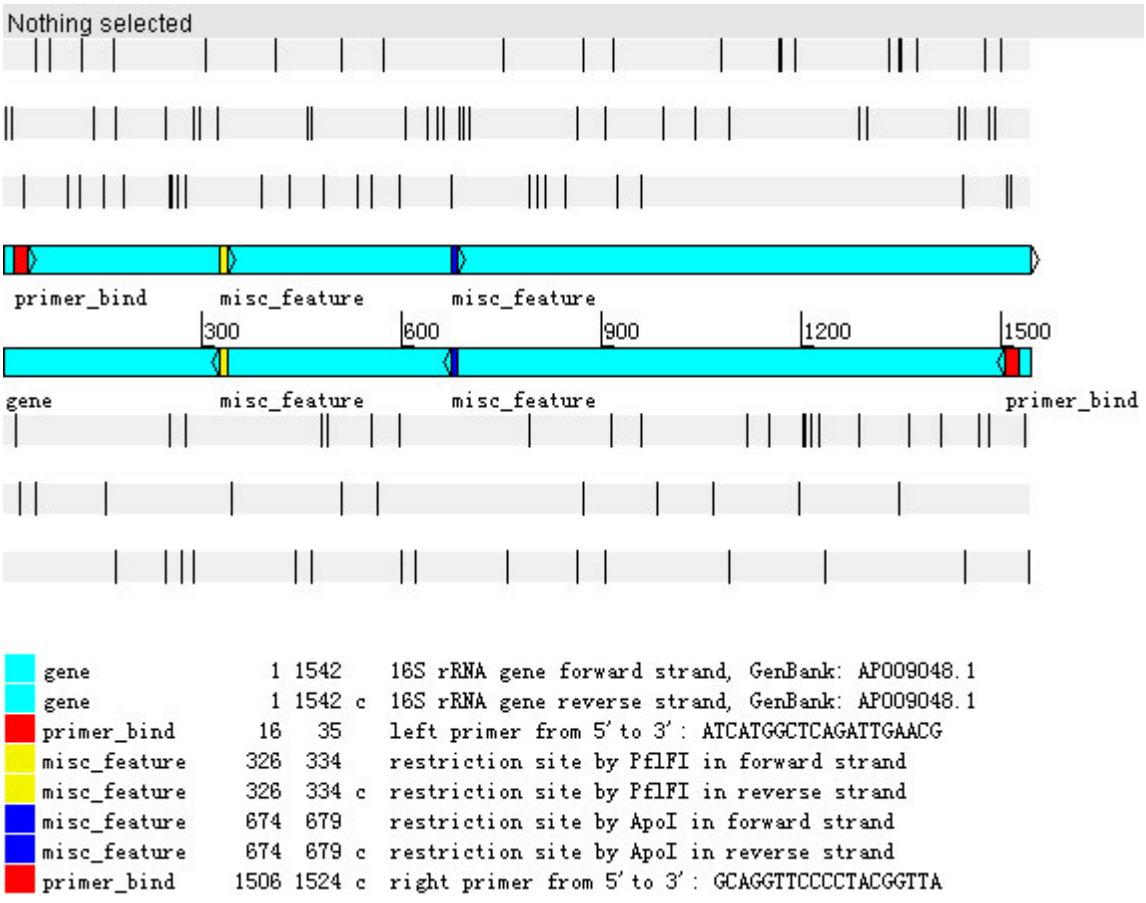
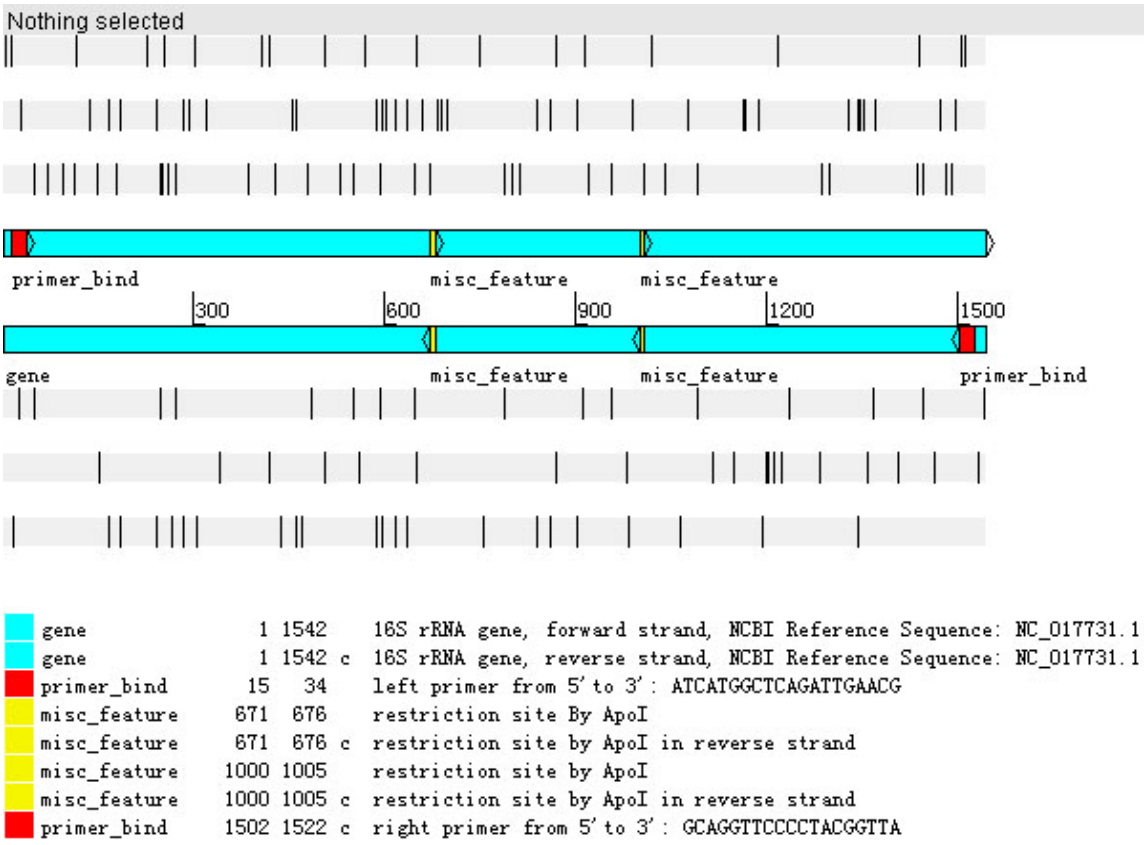


Figure 3. Complete 16S rRNA gene sequence of *E. coli* str. K-12 strain (genebank: AP009048) with one PflFI restriction sites. The light blue indicated *E. coli* str. K-12 strain (genebank: AP009048). PflFI restriction sites were indicated as yellow, ApoI restriction sites were indicated as dark blue and primers' binding sites were indicated as red. Left primer from 5' to 3': ATCATGGCTCAGATTGAACG; Right primer from 5' to 3': GCAGGTTCCCCTACGGTTA.



gene	1	1542		16S rRNA gene, forward strand, NCBI Reference Sequence: NC_017731.1
gene	1	1542	c	16S rRNA gene, reverse strand, NCBI Reference Sequence: NC_017731.1
primer_bind	15	34		left primer from 5' to 3': ATCATGGCTCAGATTGAACG
misc_feature	671	676		restriction site By ApoI
misc_feature	671	676	c	restriction site by ApoI in reverse strand
misc_feature	1000	1005		restriction site by ApoI
misc_feature	1000	1005	c	restriction site by ApoI in reverse strand
primer_bind	1502	1522	c	right primer from 5' to 3': GCAGGTTCCCCTACGGTTA

Figure 4. Complete 16S rRNA gene sequence (indicated as light blue) of *P. stuartii* MRSN 2154 (genebank: NC_017731.1) with no PflFI and two ApoI restriction sites. ApoI restriction sites were indicated as yellow and primers' binding sites were indicated as red. Left primer from 5' to 3': ATCATGGCTCAGATTGAACG; Right primer from 5' to 3': GCAGGTTCCCCTACGGTTA.

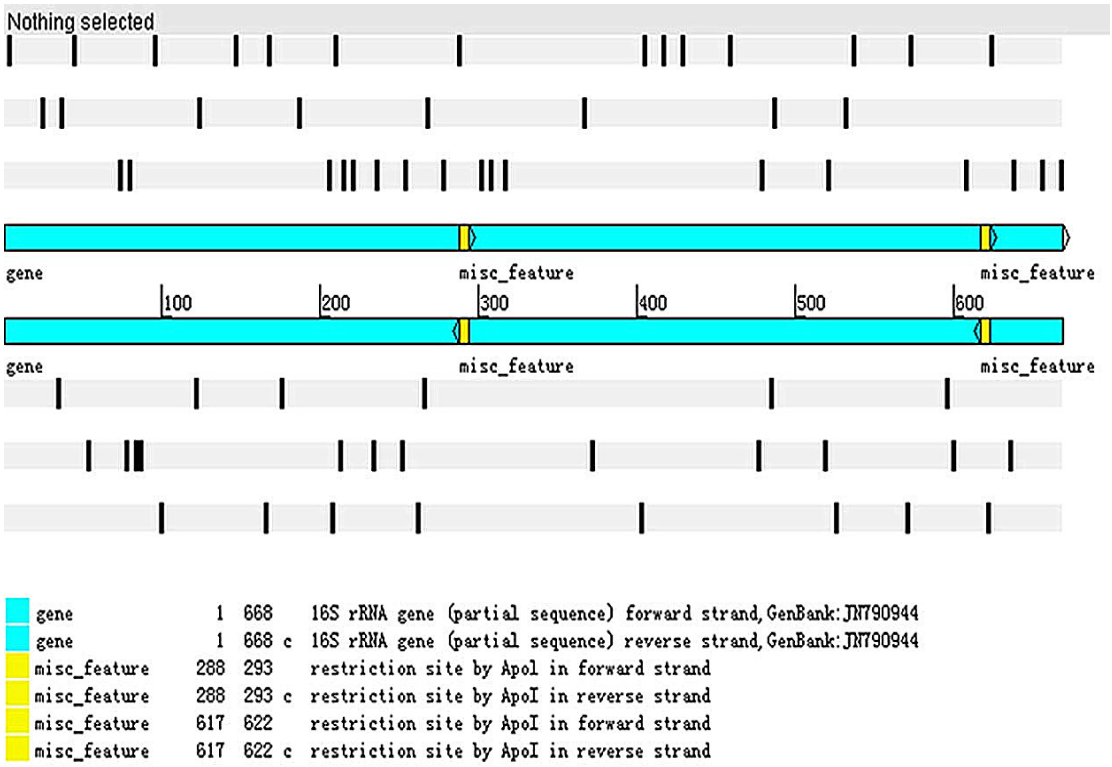


Figure 5. Partial 16S rRNA gene sequence of *Providencia* sp. Sal2, (partial sequence, accession number JN790944), with ApoI restriction sites and primers' binding sites. ApoI restriction sites were indicated as yellow. Left primer from 5' to 3': ATCATGGCTCAGATTGAACG; Right primer from 5' to 3': GCAGGTTCCCCTACGGTTA.

Size analysis for 16S rRNA gene PCR products, which were amplified by designed primers, was then carried out by electrophoresis and gel analysis, using Hi-Lo DNA ladder with a wide range of 50 to 10,000 base pairs (Minnesota Molecular Inc, Minnesota, USA) to estimate the size of the bands. All *gfp*-tagged strains (A2,A3,B1,C2,D5,D7,E7,E9,G4,H5,H6,J11), *E. coli* pUT*gfp*2 (S17-1 λ*pir*) and

Providencia sp., of which the 16S rRNA gene PCR products were all around 1.5kb (Figure 6), which were consistent with expected result by reference sequences (Figure 3 and Figure 4). Since partial sequence of 16S rRNA gene of positive control *Prov*. Sal were known [67], and it was certain that this known partial sequence have two cutters by Apol bsed on its known partial sequence (Figure 5).

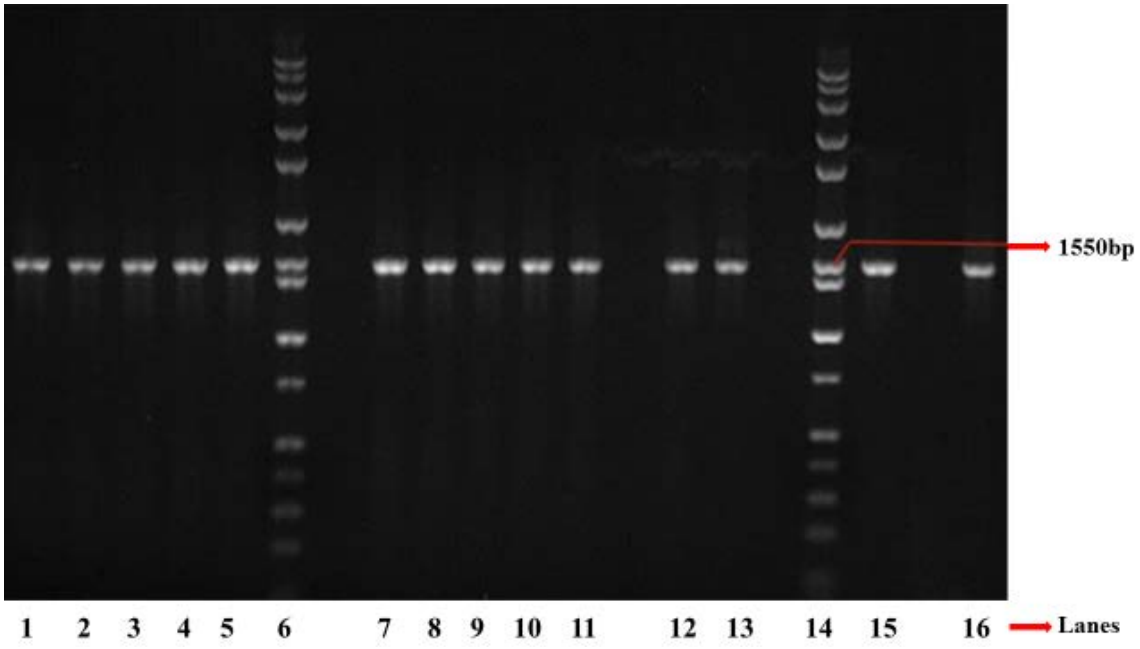


Figure 6. 16S rRNA gene PCR products of *gfp*-tagged strains, *E. coli* pUT*gfp*2 (S17-1 λ*pir*) and *Providencia* sp. Sal2. Lane 1-5, 7-11 and 12-13 indicate *gfp*-tagged *Providencia* strains of (A2,A3,B1,C2,D5,D7,E7,E9,G4,H5,H6,J11) consecutively, lane15 indicates 16S rRNA gene PCR product of *Prov*.sal2. Lane 16 indicates 16S rRNA gene PCR product of *E. coli* pUT*gfp*2 (S17-1 λ*pir*). Lane 6 and 14 indicate DNA ladder with size range of 50 bp to 10kb (Minnesota Molecular Inc, Minnesota, USA). The bands were all around size 1.5Kb according to Hi-Lo DNA marker (Minnesota Molecular Inc, Minnesota, USA).

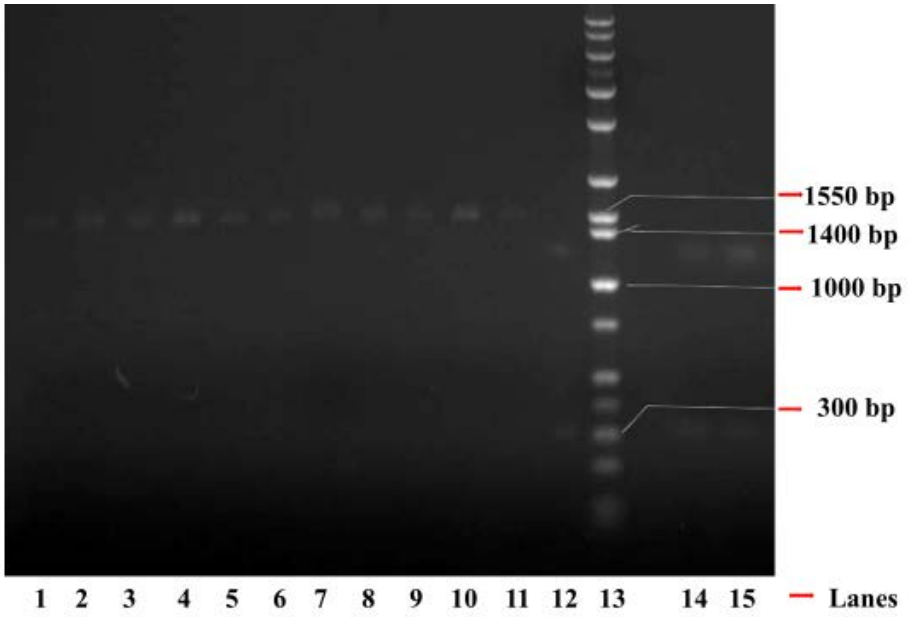


Figure 7. Restriction patterns by PflFI. From lane 1-10 indicate restriction patterns of *gfp*-tagged *Providencia* strains of A3,B1,C2,D5,D7,E7,E9,G4,H5,H6 by PflFI. Lane 11 indicated restriction pattern of *Prov.* Sal, which was not cut by PflFI. Lane 14 and 15 indicate restriction patterns of *E. coli* pUT*gfp*2 (S17-1 λ*pir*) by PflFI. *E. coli* pUT*gfp*2 (S17-1 λ*pir*) was cut once by PflFI, resulted in two bands with the size around 1000-1400bp and 300bp. Lane 13 indicates DNA ladder with size range of 50 bp to 10kb (Minnesota Molecular Inc, MN, USA).

After size confirmation of the 16S rRNA gene PCR products, confirmation of these *gfp*-tagged strains was carried out to exclude *E. coli* pUT*gfp*2 (S17-1 λ*pir*) or any other potential bacterial contamination. By using enzyme PflFI, which was expected to cut *E. coli* only, the digested fragments were examined in gel image as showed in Figure 7. Digested fragments of Positive control *Prov.* Sal were indicated in lane 11, with the constructed *gfp*-tagged *providencia* as indicated from lane 1 to lane 10 were uncut, and the size remained the same as the previous PCR products (Figure 6), indicating these *gfp*-tagged strains (from *gfp*-tagged A2 to *gfp*-tagged H6) were not *E. coli* pUT*gfp*2

(S17-1 λ*pir*). *E. coli* pUT*gfp*2 (S17-1 λ*pir*) as indicated in lane 14 and 15 showed two bands resulted from digestion with the size around 1000-1400bp and 300bp, which were consistent with the expected sizes based on the reference sequence.

Using ApoI restriction enzyme, tested samples (A3,B1,C2,D7,E7,E9,G4,H6) together with positive control showed four bands in the gel image (Figure 8), and the banding patterns were identical, the fragments of which contained 1st band with size around 750-1000bp, second and third band with size 500-750bp each, and fourth band with size 300-400bp. Negative control *E. coli* pUT*gfp*2 (S17-1 λ*pir*) showed two band, of which one band was 750bp-1000bp and the other was 500bp-750bp, which was consistent with the expected sizes of restriction sequences (Figure 3).

All the *gfp*-tagged strains were tested together with positive and negative control (Figure 8). The negative control and *gfp*-tagged strain J11 showed similar restriction patterns consisting of two bands, of which one was 750bp-1000bp and the other was 500bp-750bp. *gfp* tagged samples of A2, A3, B1, C2, D5, D7, E7, E9, G4, H5, H6 showed similar patterns, of which the 1st band with size around750-1000bp, second and third band with size 500-750bp each, and fourth band with size 300-400bp.

Since partial sequence of 16S rRNA gene of positive control *Prov*. Sal were known [67], and it was certain that this known partial sequence have two cutters by Apol (Figure 5). Instead of two cutters by ApoI in the 16S rRNA gene PCR product as indicated by the reference strain, the four-band digestion pattern for the positive control and all the tested samples showed in the result indicated another unknown restriction site somewhere outside of the known partial reference sequence.

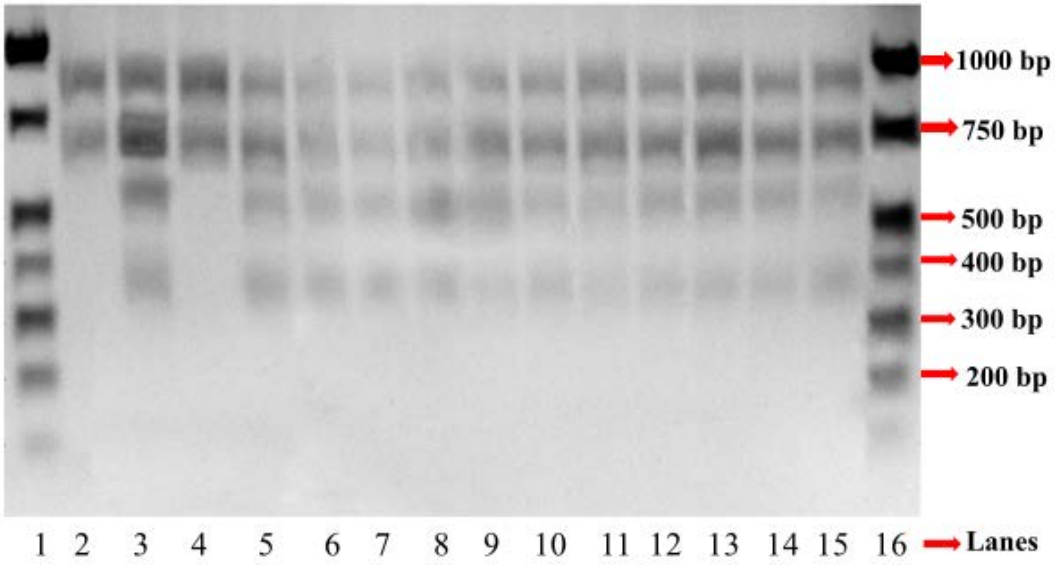


Figure 8. Restriction enzyme patterns of all *gfp*-tagged strains (fromA2 to J11), *Prov.* Sal and *E. coli* S17-1 λ*pir* by ApoI. Lane 1 indicates HiLO DNA ladder used for size determination (Minnesota Molecular Inc, Minnesota, USA). Lane 2 indicates the negative control, which is the restriction patterns of *E. coli* pUT*gfp*2 (S17-1 λ*pir*). *E. coli* pUT*gfp*2 (S17-1 λ*pir*) was cut once, resulted in two bands with the size around 750bp-1000bp and 500bp-750bp. Lane 3 indicates the positive control, which is the restriction patterns of *Prov.* Sal. Lane 4 indicates restriction patterns of J11, which showed two bands, the same as with negative control. Lane 5 to 15 indicates restriction patterns of *gfp*-tagged strains from A2 to H6, which all showed four bands, and two of them were around 500-750bp, one was 750bp-1000bp, and the other was 300-400bp.

Discussion

Documented studies demonstrate the interkingdom interactions between *L. sericata* and its commensal bacteria *P. mirabilis* [85]. It is worth noting that *Providencia* and *Proteus* are closely associated historically [163] and they have been recurrently co-isolated from *L. sericata* [85]. However the association of *Providencia* spp. with *L. sericata* is still understudied. In the study, I constructed and confirmed 11 *gfp*-tagged

Providencia strains. The construction of *gfp*-tagged *Providencia* strains was achieved through bi-parental mating by *Providencia* spp. with *E. coli* pUT*gfp*2 (S17-1 λ*pir*), in which donor *E. coli gfp*2 S17-1 λ*pir* delivered the antibiotic resistance marker Kan ^{r} and *gfp* gene into recipient *bacteria Prov. Sal*. I designed primer pairs that were able to amply the entire 16S rRNA gene of *Prov*. Sal and *E. coli gfp*2 S17-1 λ*pir*.

The construction of *gfp* gene in *Providencia* will provide tools to investigate interactions between *Providecia,* which is one of the most abundant bacteria isolated inside of *L. sericata*, with *L. sericata* immature and adults, as well as the effects on this bacteria the adult's potential choice for food location and oviposition. The physiological responses of *L. sericata* immature and adults after exposure to the *Providencia strains* will further lead to the identification of genes *in L. sericata* that function in interkingdom interactions with *Providencia* spp.

Providencia, as a commensal exist inside of *L. sericata,* indicates potentially involvement in the process of development as well as the behavior of this insect. Understanding the inter-kingdom communication between *L. sericata* and *Providencia* will be useful for further discovering new QS molecules and related gene expressions involved in the process. The bacteria distribution and the degree of infection and change of bacteria load will be measured after ingestion by larvae, to investigate into the resistance of the bacteria as a commensal inside of this insect. Understanding this interaction will help to find ways to control or inhibit the vector competence for pathogens, as well as find ways to interrupt the interaction between the insects and the

potential pathogens, which will be useful to manage and prevent the health hazard caused and spread by their interactions.

In summary, the current research provides a reliable method for construction *gfp*-tagged *Providencia* strains from wild type *Provi*den*cia* sp., as well as for identification and differentiation of *Prov*. Sal and *E. coli gfp*2 S17-1 λ*pir*. The constructed *gfp*-tagged *Providencia* will be useful for further study the interactions of this bacteria and its host insect.

CHAPTER III

SEX AND OVARIAN STATUS INFLUENCE RESPONSE OF *LUCILIA SERICATA* (MEIGEN) (DIPTERA: CALLIPHORIDAE) TO VARIOUS DOSES OF COMPOUNDS RELATED TO LARVAL RESOURCES

Introduction

Interactions between insect and microbes are very frequent due to the cosmopolitan distribution of both of them. Documented research studied the interkingdom interactions between *L. sericata* and its commensal bacteria, *Proteus*. In the previous chapter I used a wild type *Providencia* that was previously isolated from *L. sericata* to construct a *gfp*-tagged *Providencia*, which can be used for further study of interactions between *L. sericata* and this potential commensal bacteria. However, interactions between *L. sericata* and bacteria through insect chemoreception of bacterially produced VOCs are relatively neglected.

Microbes play an important role in the decomposition processes of carrion as well as other materials such as feces, urine, animal secretions (e.g., sweat), decomposing plant material, fungi and algae [67]. Through the decomposition process, high-molecular-weight nutrients break down to their constituents. For example, proteins degrade into sulphur, nitrogen, and phosphorus-containing compounds [176]. The volatile organic compounds (VOCs) produced by bacteria represent many of the compounds that serve as cues regulating attraction or repellence of insects to these resources [67]. They are essential for flies to recognize potential mates and kin,

oviposition sites and food sources [67]. Numerous necrophagous species feed on the same remain resource [149]; thus, competition also exist between microbes and associated insects attempting to exploit the same resources through regulation of microbial VOCs [177-179]. Thus it is possible that the carrion-feeding organisms may have evolved strategies to exploit the remains before it is fully consumed by competitors [149].

VOCs released from decomposing vertebrate remains attract a wide range of insects [129-132]. *L. sericata* is an example of a typical arthropod that is an early colonizer of carrion [149]. Consequently, these insects have been selected to have highly sensitive olfactory systems enabling them to detect low levels of VOCs indicative of the presence of carrions sources [180-182].

In this chapter I used VOCs that are associated with blow flies and decomposing vertebrate carrion, dimethyldisulfide (DMDS), phenylacetic acid (PAA), indole, and isobutylamine to study the olfactory response of *L. sericata* to carrion associated resource.

In previous work, *P. mirabilis* was isolated from *L. sericata* salivary glands and by using transoposon mutagenesis, a series of *P. mirabilis* mutants with decreased swarming ability were constructed [67]. Tomberlin et al. [74] used one of these mutant strains deficient in swarming and rescued by putrecine to test the effect on attraction and oviposition of *L. sericata* and they determined the *Proteus* mutant was less attractive to *L. sericata* attraction and oviposition compared to wild type strain [74]. Gas chromatography mass spectrometry (GC-MS) of the mutant and wild type strains

indicated that compounds that emitted from the wild type *Proteus* and this mutant strain were quite different with respect to all of the aforementioned molecules, with imethyldisulfide (DMDS), Phenyletheyl alchohol, indole, and isobutylamine being the greatest difference between wild type and the mutant strain [74].

While examining the responses of *L. sericata* to the mutant and wild type *P. mirabilis*, Tomberlin et al., [74] also determined that response was governed by sex with males and females responding differently to the bacteria [74]. This behavior is not unique to blow flies. Sexual differences of response to odors has also been documented for the fruit fly *Ceratitis cpitata* (Wiedemann) (Diptera: Tephritidae) in which males are more responsive than females [145]. Furthermore, female house flies, *Musca domestica* L., (Diptera: Muscidae) are more strongly attracted to ammonia than males and vice versa with respect to ethyl alcohol [138].

It is also known that different sexes respond differently to VOCs of different types and different doses. For *D. melanogaster*, when exposed to ethyl alcohol, adult females showed a higher response than male flies in response to ethyl alcohol (up to concentration of 25%). When exposed to acetic acid, *Drosophila* females demonstrated higher responsiveness to males in response to the compound at a 1% concentration. In addition to sex, ovarian status influences insect responses to VOCs [74].

As discussed in previous chapters, DMDS, PAA, indole and isobutylamine are by-products derived from the essential amino acids of methionine, tryptophan, phenylalanine and valine, respectively, which in many cases cannot be synthesized *de novo* by many higher organisms. *Proteus* and *Providencia* spp. are among the microbes

that break down, as well as and synthesis methionine, and they are the two bacteria that had been recurrently isolated from *L. sericata* salivary gland. I thus contend that the responsiveness of blow fly to these VOCs may be related with the fact that 1) blow flies use the odorous cues to direct/locate carrion resource so as to obtain the nutrition from dead animal tissue or from bacteria that cannot be produced by themselves. My hypothesis was that the response of *L. sericata* to carrion associated VOCs will differ based on their different sex and ovarian status of the flies.

Methods

Colony maintenance

L. sericata adults were maintained in 30 x 30 x 30 cm^2 BioQuip bug dorms (Bioquip Products, Rancho Dominguez, CA, USA) [183]. Adult flies were fed bovine liver blood *ad libitum* for the first 5 day post emergence in order to stimulate ovarian development. Flies were then fed sugar and water until testing at ages 7-9 day.

In order to maintain the colonies, flies remaining in the cages after day 9 were provided bovine liver in 88.7 ml plastic bath cups (Wal-mart.Inc., Bentonvillle, AR, USA) for 2-3 days. Resulting eggs and liver were placed into 900 ml mason jars. The bottom 5 cm of the jars was filled with sawdust. Larvae dispersing from the liver were allowed to pupate in the saw dust. Resulting adults were managed using methods previous described.

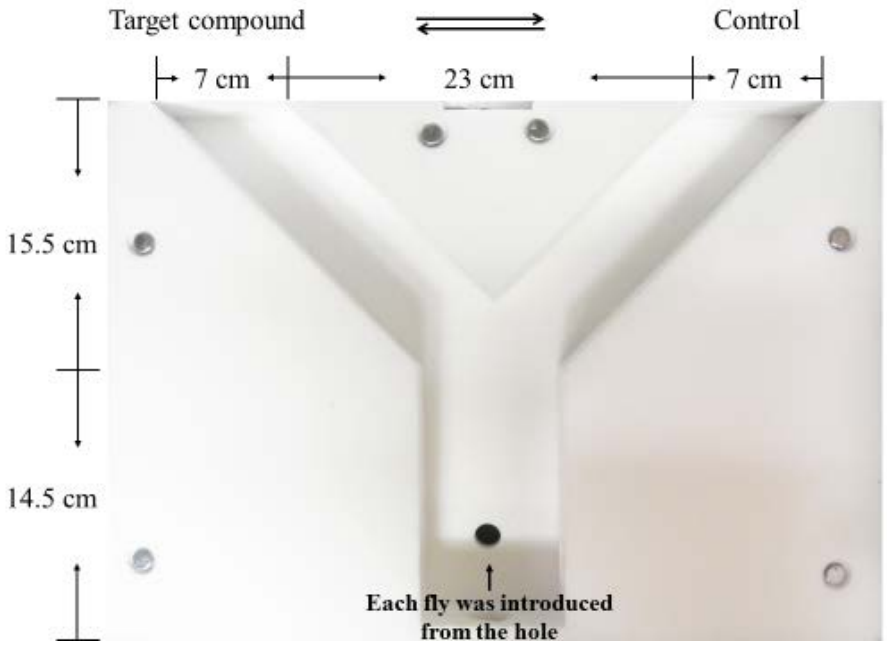


Figure 9. Dual choice olfactometer for behavioral response of *L. sericata* adults exposed to treatments. Each fly was introduced into the system and was given three minutes to make choices .The residence time each fly spent on each arm was recorded for analysis.

Y-tube olfactometer assay

A dual choice olfactometer was used to evaluate the behavioral response of *L. sericata* adults exposed to treatments (Figure 9). The olfactometer was covered with a removable sheet glass. A 50 mm USB powered computer cooling fan (5VDC Fan, Dc Fans, Thermal Management NMB Technologies Corporation, Chatsworth, CA, USA) was used to pull air through the olfactometer. Two 15 mm diameter, 14.5 cm long glass tubes containing activated charcoal (Aqua-Tech, Marineland Aquarium Products,

Moorpark, CA, USA) were attached to the ziploc containers, which were used to hold the treatments for all experiments, to clean the air flowing through the olfactometer. Containers were attached to olfactometer arms with 14 Tygon tubing, and the containers were rotated after the completion of each experiment. Fluorescent light served for overhead illumination. Olfactometer assay was carried out with temperature between 20ºC - 23ºC. The Y-tube was cleaned after each test with 80% ethanol and allowed to air dry for 2 min subsequent experiments. Ten µL of each tested solution was applied in filter paper in the shape of circular sector ($\theta = 22.5°$, radius 4.5 cm) (Fisher Scientific, 81 wyman street Waltham MA, USA) and allowed 5 min for the solvent to evaporate in the ventilation hood before putting into each container. Treatment location in the arms of Y-tube was rotated between replicates to rule out any bias for any side of the arm. Newly treated filter papers were used for each insect. Each fly was introduced into Y-tube and allowed to spend 3 min in the olfactometer.

DMDS (Sigma Aldrich, Basic materials, St. Louis, MO, USA, Purity ≥99.0%) was diluted with acetone to concentrations of 25 µg/ml, 5 µg/mL, 0.5 µg/mL, and 0.05 µg/mL. For the experiments, 10 µL of each diluted DMDS solution was applied to a filter paper. For the control, 10 µL of acetone was applied to the filter paper.

The methods described above were also used to prepare other chemicals. indole (Sigma Aldrich, Basic materials, St. Louis, MO, USA, purity ≥ 99.0%) was prepared in a series of doses at 0.05, 0.5 and 5µg; PAA (Sigma Aldrich, Basic materials, St. Louis, MO, USA , purity ≥ 99.0%) was prepared to make a series of doses at 0.1 µg, 1 µg and

10 μg; isobutylamine (Sigma Aldrich, Basic materials, St. Louis, MO, USA, purity≥ 99.0%) was prepared to make a series of doses at 0.01 μg, 0.1μg and 1 μg.

Twenty male, gravid, and non-gravid females of 7-9 d post emergence were tested for each treatment. Each individual fly was introduced into the Y-tube at the entrance of the main branch and had a choice between the treatment pairs. If a fly remained within the stem of the olfactometer 30 s after being introduced, it was replaced. Total residence time in each arm was recorded for each fly.

Statistical analysis

The residence time data was analyzed with PROC MIX (SAS 2011) using a full factorial design. The statistical model tested evaluated fixed factors that included the Type (indicates the types of the flies based on their sexes and ovarian development), Test (indicates responses to control or treatments), Dose (indicates different doses of the compounds), and the 2-way and 3-way interactions of these factors. Least square means was used to evaluate statistical differences among treatments ($P < 0.05$). In addition, flies were categorized as gravid females (GF), non-gravid females (NG) and males (M).

Results

The model with the interactions (Dose *Test* Type) in the model is a slight improvement over the model which deletes this term. For example, the AIC (Akaike information criterion) is smaller in the model with the 3-way interaction included versus the model without this term (Table 2).

Type 3 tests showed that fly responses were marginally different in response to control and DMDS in regards to different doses of DMDS and classes (different sexes and ovarian status) (F1, 2 = 2.08, df1 = 6, df2 = 480, P = 0.0537) (Appendix A-1). The probability of response ± SE of 7-9 days old *L. sericata* adults of different types (sexes and ovarian status) to different doses of DMDS was demonstrated in Table 4.

Table 2. Fit Statistics of models for testing blow fly response to VOCs

	Model without 3-way interactions	Model with 3-way interactions
-2 Res Log Likelihood	5599.5	5448.7
AIC (Akaike information criterion)	5601.5	5450.7
AICC (small-sample-size corrected version of Akaike information criterion)	5601.5	5450.7
BIC (Bayesian information criterion)	5605.7	5454.9

Table 3. Differences of Least Squares Means. Fly of different types in response to different VOCs were compared. GF: gravid females, NG: non-gravid females, M: males.

VOCs	Effect	Dose	Type	Tests	vs	Dose	Type	Test	Pr > |t|
DMDS	Dose*Type*Test	0.005	GF	treatment	vs	0.005	M	treatment	0.0037
indole	Dose *Test	0.05		control	vs	5		control	0.0091
indole	Dose *Test	0.05		treatment	vs	5		treatment	0.0067
indole	Dose *Test	5		control	vs	5		Treatment	0.0012
PAA	Test			control	vs			treatment	0.0123
PAA	Sex*Test		GF	control	vs		GF	treatment	0.0022
PAA	Sex*Test		NG	control	vs		NG	treatment	0.0169
PAA	Dose*Type*Test	10	GF	control	vs	10	GF	treatment	0.0014
isobutylamine	Type*Test		GF	Treatment	vs		M	treatment	0.0121
isobutylamine	Type*Test		M	Control	vs		M	treatment	0.0188
isobutylamine	Type*Test		M	Control	vs		NG	control	0.0053
isobutylamine	Type *Test		M	Treatment	vs		NG	treatment	0.0151
isobutylamine	Type *Test		NG	control	vs		NG	treatment	0.0041
isobutylamine	Dose* Type *Test	0.01	M	control	vs	1	M	control	0.0027
isobutylamine	Dose* Type *Test	0.1	NG	control	vs	0.1	NG	treatment	0.0059
isobutylamine	Dose* Type *Test	0.01	M	control	vs	0.01	M	treatment	0.0025

Table 4. Probability of response ± SE of 7-9 days old *L. sericata* adults of different types (sexes and ovarian status) to different doses of DMDS.

VOCs	Doses (µg)	Type	Probability of response to treatments ± SE (%)	Probability of response to blank ± SE (%)
DMDS	0.2500			
		GF (20)	55.88 ± 8.96	45.12 ± 8.96
		NG (20)	66.18 ± 16.40	33.82 ± 16.40
		M (20)	45.42 ± 10.10	54.58 ± 10.10
	0.0500			
		GF (20)	46.41 ± 9.69	53.59 ± 9.69
		NG (20)	44.67 ± 9.32	55.33 ± 9.32
		M (20)	46.72 ± 10.32	53.28 ± 10.32
	0.0050			
		GF (20)	26.38 ± 8.61	73.62 ± 8.61
		NG (20)	47.67 ± 8.32	62.33 ± 8.32
		M (20)	64.57 ± 9.42	35.43 ± 9.42
	0.0005			
		GF (20)	49.81 ± 8.26	50.19 ± 8.26
		NG (20)	43.41 ± 8.10	56.59 ± 8.10
		M (20)	49.34 ± 10.04	50.66 ± 10.04

In response to DMDS at 0.005 µg ($F_{1, 2}$ = 2.92, df_1 = 6, df_2 = 480, P = 0.0037), gravid females were repelled while males were attracted to the compound (Table 3 and Table 4). However, response to 0.05 µg of DMDS did not show significant attraction in individual class in the current study, which is in contrast to Frederickx et al. [77]. Instead, my result showed that DMDS attracted males and repelled gravid females at the dose of 0.005 µg, and the responses difference were statistically significant ($F_{1, 2}$ = 2.92, df_1 = 6, df_2 = 456, P = 0.0037) (Table 3, Figure 10).

Table 5. Probability of response ± SE of 7-9 days old *L. sericata* adults of different types to different indole doses.

VOCs	Doses (µg)	Type	Probability of response to treatments ± SE (%)	Probability of response to blank ± SE (%)
Indole	5			
		GF (20)	64.67 ± 7.93	35.33 ± 7.93
		NG (20)	64.11 ± 9.36	35.99 ± 9.36
		M (20)	66.98 ± 6.39	33.02 ± 6.39
	0.5			
		GF (20)	42.83 ± 8.05	57.17 ± 8.05
		NG (20)	57.97 ± 9.89	42.03 ± 9.89
		M (20)	49.71 ± 10.95	50.29 ± 10.95
	0.05			
		GF (20)	47.88 ± 9.84	53.12 ± 9.84
		NG (20)	38.19 ± 9.36	61.81 ± 9.36
		M(20)	34.55 ± 9.75	65.46 ± 9.75

Table 6. Probability of response ± SE of 7-9 days old *L. sericata* adults of different types to different doses of PAA.

VOCs	Doses (µg)	Type	Probability of response to treatments ± SE (%)	Probability of response to blank ± SE (%)
PAA	10			
		GF (20)	72.08 ± 9.41	28.92 ± 9.41
		NG (20)	58.13 ± 7.96	41.87 ± 7.96
		Male (20)	41.12 ± 10.13	58.88 ± 10.13
	1			
		GF ((20)	55.88 ± 9.36	44.12 ± 9.36
		NG (20)	55.35 ± 8.43	44.65 ± 8.43
		M (20)	49.82 ± 9.36	50.18 ± 9.36
	0.1			
		GF (20)	59.93 ± 6.53	40.07 ± 6.53
		NG (20)	62.41 ± 11.07	37.59 ± 11.07
		M (20)	42.805 ± 10.32	57.20 ± 10.32

Based on type 3 tests of fixed effects, regardless of sex, all flies showed significantly different response to indole and control in regard to different doses ($F_{1, 2}$ = 7.17, df_1 = 2, df_2 = 342, P = 0.0009) (Appendix A-1). Besides, flies, regardless of sex and ovarian status, all showed significant attraction in response to indole at dose 5 µg (Table 3 and Figure 11). The probability of response ± SE of 7-9 days old *L. sericata* of different types to different doses of indole was demonstrated in Table 5.

Regardless of doses, flies of different sexes and ovarian status responded differently to PAA (F1, 2 = 4.4, df1 = 2, df2 = 358, P = 0.0130) (Appendix A-1). Nongravid and gravid females all showed significant attraction to PAA within the tested doses range (Table 3 and Figure 12). The probability of response ± SE of 7-9 days old *L. sericat*a of different types to different doses of PAA was demonstrated in Table 6

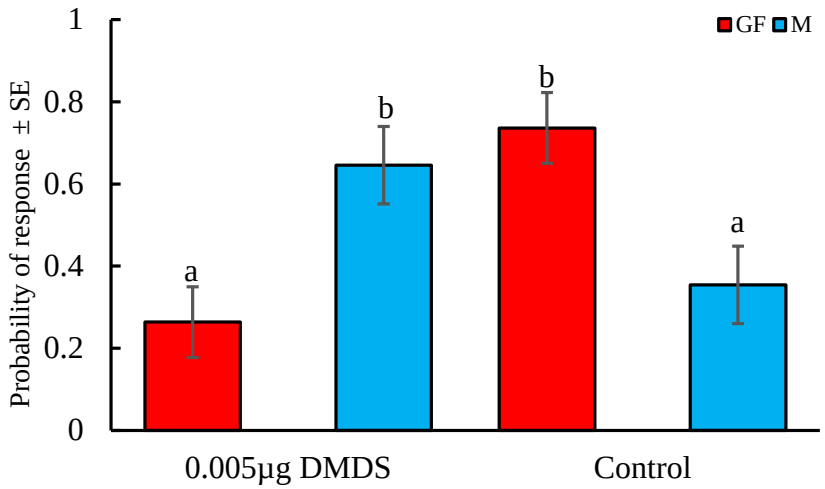


Figure 10. Probability of response ± SE of *L. sericata* adults (7-9 d-old) to different DMDS at a dose of 0.005 µg vs control in a Y-tube olfactometer. Dose * type * treatment effect was significant. Different letters indicated significant difference ($F_{1, 2} = 2.92$, $df_1 = 6$, $df_2 = 456$, $P < 0.05$).

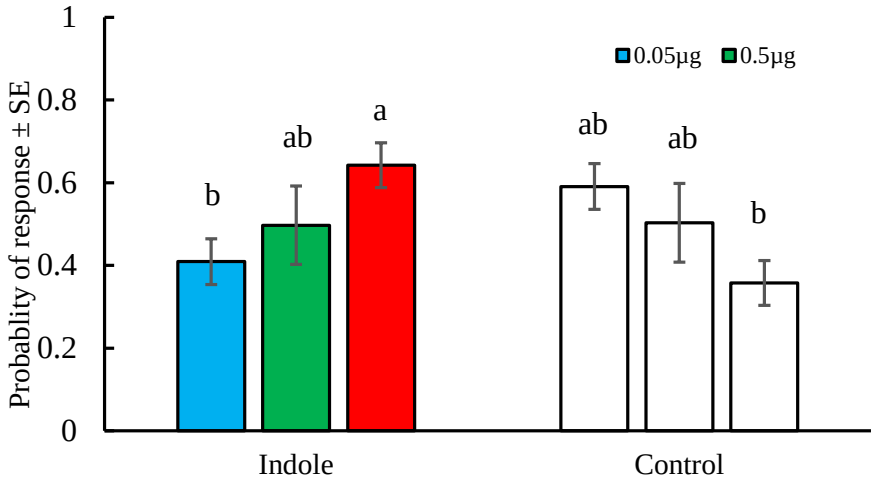


Figure 11. Probability of response ± SE of *L. sericata* adults (7-9 d-old) to different indole doses. Probability of response ± SE of *L. sericata* adults (7-9 d-old) to different indole at dose 0.05 µg vs 5 µg was significant ($F_{1, 2} = 2.73$, $df_1 = 2$, $df_2 = 342$, $P = 0.0067$); Probability of of response ± SE of *L. sericata* adults (7-9 d-old) to indole at dose 5 µg vs control was significant ($F_{1, 2} = 3.27$, $df_1 = 2$, $df_2 = 342$, $P = 0.0012$). Different letters indicated significant difference.

Response to isobutylamine were significantly differed between control and treatments ($F_{1, 2} = 2.45$, $df_1 = 2$, $df_2 = 342$, $P = 0.0459$) (Appendix A-1). Besides, Gravid and non-gravid females all showed higher responses to isobutyl amine when compared with males; Non gravid females were attracted to isobutylamine at 0.10 µg (Figure 13), and males showed significant repellence to isobutylamine at 0.01 µg (Figure 13). Probability of response ± SE of 7-9 days old *L. sericata* of different sexes to different isobutylamine doses were demonstrated in Table 7.

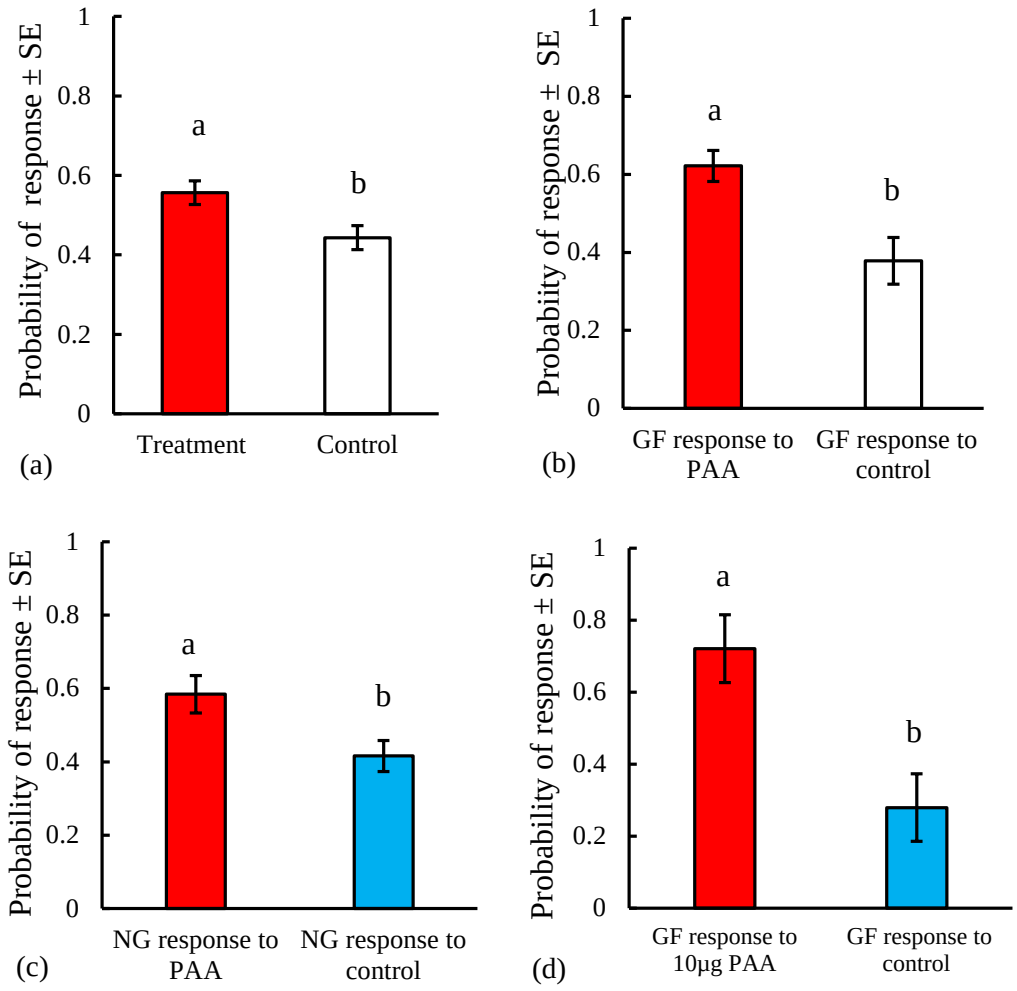


Figure 12. Mean percentage of response time of all *L. sericata* adults (7-9 d-old) to different PAA doses. a: Mean percentage of response time of all *L. sericata* adults (7-9 d-old) to PAA within tested dose range of 0.1-10 µg vs control in a Y-tube olfactometer ($F_{1,2} = 2.52$, $df_1 = 1$, $df_2 = 342$, $P = 0.0123$.) b: Mean percentage of response time of *L. sericata* gravid females (7-9 d-old) to PAA dose range of 0.1-10 µg vs control ($F_{1,2} = 3.08$, $df_1 = 2$, $df_2 = 342$, $P = 0.0022$) c: Mean percentage of response time of *L. sericata* non gravid females (7-9 d-old) to PAA dose range of 0.1-10 µg vs control ($F_{1,2} = 2.4$, $df_1 = 2$, $df_2 = 342$, $P = 0.0169$) d: Mean percentage of response time of all *L. sericata* adults (7-9 d-old) to PAA at dose 10 µg vs control ($F_{1,2} = 3.23$, $df_1 = 4$, $df_2 = 342$, $P = 0.0014$). Different letters indicated significant difference.

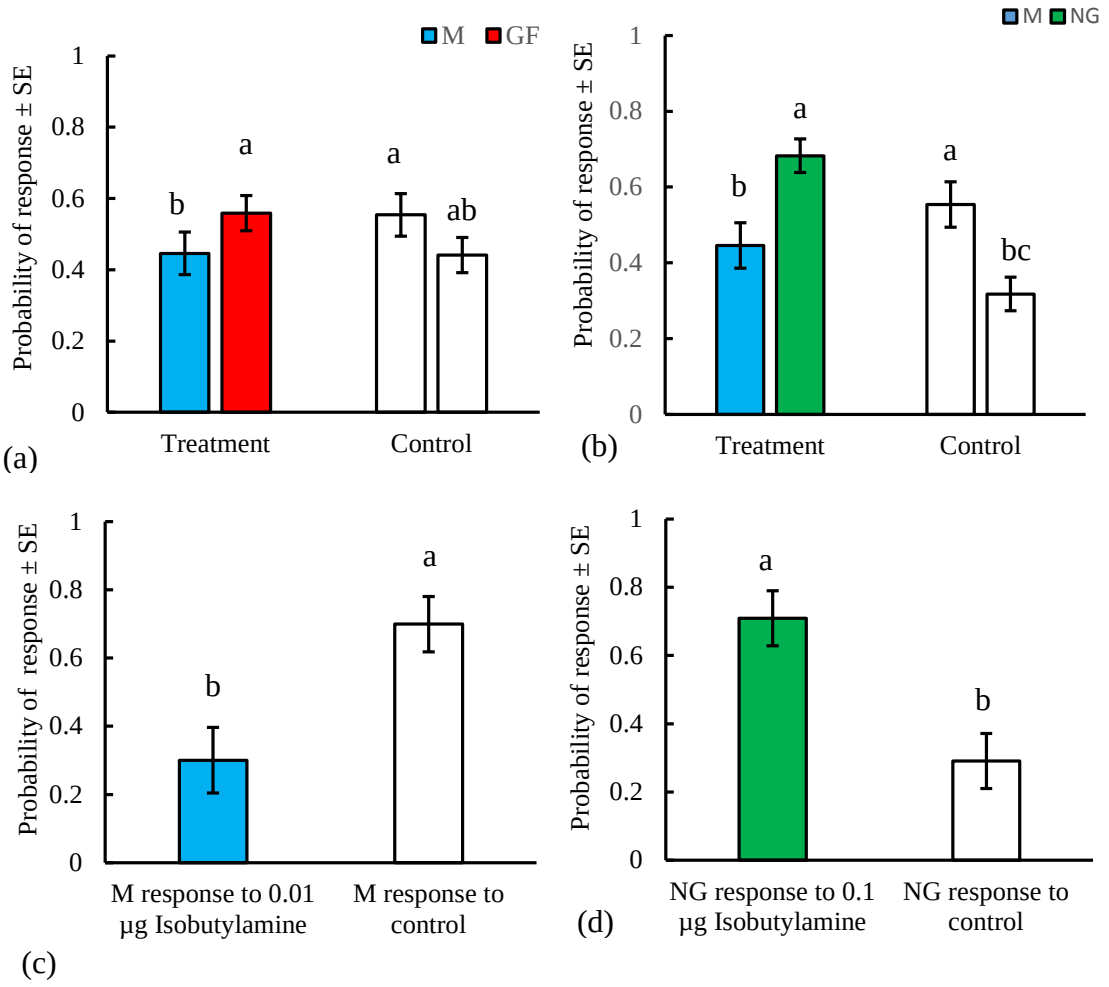


Figure 13. *L. sericata* adults (7-9 d-old) in response to different isobutylamine doses. (a) Probability of male and gravid females *L. sericata* in response to isobutylamine within tested dose range of 0.01-1 µg vs control in a Y-tube olfactometer. Probability significantly differed between males and gravid females in response to isobutylamine ($F_{1, 2} = 2.52$, $df_1 = 2$, $df_2 = 342$, $P = 0.0121$); Probability significantly differed between response of males to isobutylamine vs control ($F_{1, 2} = 2.36$, $df_1 = 2$, $df_2 = 342$, $P = 0.0188$). M indicates males, GF indicates gravid females. (b) Probability of male and non-gravid females in response to isobutylamine within tested dose range of 0.01-1 µg vs control. Probability significantly differed between males and non-gravid females in response to isobutylamine ($F_{1, 2} = 2.81$, $df_1 = 2$, $df_2 = 342$, $P = 0.0053$); Probability of males significantly differed between response to isobutylamine vs control ($F_{1, 2} = 2.36$, $df_1 = 2$, $df_2 = 342$, $P = 0.0188$); Probability significantly differed between response of males to isobutylamine vs control ($F_{1, 2} = 2.89$, $df_1 = 2$, $df_2 = 342$, $P = 0.0041$. M indicates males, NG indicates non-gravid females. (c) Probability of male *L. sericata* in response to isobutylamine at dose 0.01 µg vs control. Probability significant differed between response to isobutylamine vs control ($F_{1, 2} = 3.04$, $df_1 = 4$, $df_2 = 342$, $P = 0.0025$) d) Probability of non-gravid female *L. sericata* in response to isobutylamine at dose 0.1 µg vs control. Probability significantly differed between response to isobutylamine vs control ($F_{1, 2} = 2.77$, $df_1 = 4$, $df_2 = 342$, $P = 0.0059$). Different letters above the columns in the figure indicate significance ($P < 0.05$).

Response to isobutylamine were significantly differed between control and treatments (F1, 2 = 2.45, df1 = 2, df2 = 342, P = 0.0459) (Appendix A-1). Besides, Gravid and non-gravid females all showed higher responses to isobutyl amine when compared with males; Non gravid females were attracted to isobutylamine at 0.10 µg (Figure 13), and males showed significant repellence to isobutylamine at 0.01 µg (Figure 13). Probability of response ± SE of 7-9 days old *L. sericata* of different sexes to different isobutylamine doses were demonstrated in Table 7.

Table 7. Probability of response ± SE of 7-9 days old *L. sericata* adults of different sexes to different isobutylamine doses.

VOCs	Doses (µg)	Type	Probability of response to treatments ± SE (%)	Probability of response to blank ± SE (%)
Isobutylamine	1			
		GF (20)	54.98 ± 8.84	44.02 ± 8.84
		NG (20)	68.77 ± 8.08	31.23 ± 8.08
		M (20)	68.30 ± 9.63	31.70 ± 9.63
	0.1			
		GF (20)	50.02 ± 9.73	49.98 ± 9.73
		NG (20)	70.91 ± 8.03	29.09 ± 8.03
		M (20)	35.46 ± 10.16	64.54 ± 10.16
	0.01			
		GF (20)	62.68 ± 6.95	47.32 ± 6.95
		NG (20)	65.05 ± 7.29	34.95 ± 7.29
		M (20)	30.05 ± 9.64	69.95 ± 964

Discussion

Physiological status can profoundly affect the ability of an insect to detection and respond to of odorous cues [150]. Similarly, decision making by *L. sericata* is affected by nutrition [74]. Flies fed with blood showed a significant higher level of response to

wild type *P. mirabilis* than when fed with powdered milk. Nutritional status influences the physiology and egg production of flies [184, 185] and thus subsequently affects the activity of associated behaviors such as locating and selecting of resource [74]. Age also affects the selection and response of flies to resources. Resources preferences differed between older flies and younger flies, due to the different levels of selectivity of resources for locating and oviposition. Older flies tend to have lower selectivity for resources for locating food and oviposit eggs [74, 148].

Tomberlin et al.[74] found that a smaller percentage of milk-fed females was gravid compared with those fed blood from beef liver. In that experiment sex ($P < 0.0001$) has significant effects on flies choice in the olfactometer [74]. Tomberlin et al.[74] used one of these mutant strains deficient in swarming signaling capability to test the effects on attraction and oviposition of *L. sericata*. They determined the *Proteus* mutant was less attractive to *L. sericata* and yielded less oviposition compared to the wild type strain [74]. This mutant strain were quite different in its production of DMDS, indole, isobutylamine and phenylethyl alcohol compared to wild type [74], which may indicate the potential role of these differentially expressed VOCs in flies' olfactory response. Aside from the characteristics of antibiotic agent and its association with the common bacteria *Proteus* isolated from *L. sericata*, phenylacetic acid also has similarity in structure as well as sharing the same decomposing pathway with Phenylethyl alcohol [101]. Based on previous result, my research thus focused on the effect of DMDS, indole, PAA and isobutylamine on fly olfactory response.

Adult female *L. sericata* was documented to be attracted to DMDS [77], however the dose response of flies, their preferences to the tested compounds in terms of retention time in the Y-tube as well as the physiological status of tested flies remains understudied.This current research showed that flies responses were significantly different in response to control and DMDS in regards to different doses of DMDS and classes (different sexes and ovarian status) ($F_{1, 2} = 2.19$, $df_1 = 6$, $df_2 = 456$,$P = 0.0430$). In response to DMDS at 0.005 μg ($F_{1, 2} = 2.92$, $df_1 = 6$, $df_2 = 456$, $P = 0.0037$) gravid females were repelled while males were attracted. In contrast to results by Frederickx et al. [77], DMDS did not show significant attraction to flies at dose of 0.05 μg in my study. However, gravid females and males responded significantly different ($F_{1, 2} = 2.92$, $df_1 = 6$, $df_2 = 456$, $P = 0.0037$) to DMDS at dose 0.005 μg.

The current study applied full factorial design to analyze the behavioral data which allowed me to take into account the dose and sex and ovarian development effect on the response of the flies while chi-square used by Frederickx et al. [77] will not be able to analyze more than one factor in the model. Frederickx et al. [77] did not distinguish between gravid and non-gravid females in their experiments. Although the difference has not been demonstrated as statistically significance in the current study of flies' response to DMDS, it is still important to bear in mind that gravid female blow flies might respond differently to volatile organic compounds than non-gravid females. Failures to take into account of ovarian status of the tested females will likely result in discrepancy in conclusion about the response of female flies, e.g. The attraction resulting from non-gravid females will mask the repellent response from gravid females when

females are evaluated together, especially if there are more non-gravid females in the group.

Rather than just recording the first choice made by each tested flies to target chemicals [77], the dose response of flies, and their preferences to the tested compounds in terms of retention time in the Y-tube are determined in the current research. We found it more reliable to record flies' behavior within three minutes after introduction into the olfactometer than just record their first choice to the side of the treatment since flies in this experiment were allowed to make choice and decisions based on their perception of the odor cues emitted from the Y-tube, while the first choice of entering the side of Y-tube could be a transient and random activity and are less likely to reflect the actual response of flies to the tested cues.

In the context of carrion ecology, the response of *L. sericata* to the carrion resources is associated with a suite of biotic and abiotic factors, including the patterns volatile organic compounds, which may differ across carrion types and the associated microbes found on remains. The current research will provide a stepping stone for further studying response of the fly to more complicated and mixed VOCs associate with the carrion resource in the real world. Since the presence of the bacteria affect the pre-colonization interval, thus affect the estimation of the time of death of the remains. By understanding the response of the fly and the VOCs can help to predict the pattern of the bacteria on the carcass so as to have a more precise estimation of the death time of remains.

CHAPTER IV

PERFORMANCE OF LUCILIA SERICATA (MEIGEN) (DIPTERA: CALLIPHORIDAE) LARVAE ON IMBALANCED ARTIFICIAL AMINO ACID DIETS

Introduction

The previous chapter discussed the importance of VOCs in resource exploitation for *L. sericata* (Meigen) (Diptera: Calliphoridae), and discovered carrion associated VOCs affect adult fly behaviors based on their sex and ovarian states. Carrion resources are not just important for adults, but also for larval survival [149]. Once hatched out from eggs, *L. sericata* larvae have limited mobility thus are restricted in choice with regards to where they feed and obtain nutrition for growth and development. So on one side, the performance of larvae will be largely affected by adult choice of resource sites for food and oviposition, on the other side, the larval strategies for utilizing the resource are critical to survive considering the fact that carrion resource is ephemeral, lasting only a few days to a few weeks [149], and also heterogeneous in terms of space, temperature and nutrition [159]. Larvae must utilize the best resource quickly before it disappears after being consumed by other competitors (arthropods and microbes) [149]. Thus I am asking how larvae perform on carrion associated resources as a result of both maternal choice of resource and larval strategies of resource utilization.

Microbes affect maternal choice in oviposition site selection. Insects prefer to lay their eggs on microbe rich environments compare to sterile environments [128, 160].

Proteus mirabilis, a commensal isolated from *L. sericata*, attracted more adult *L. sericata* and induced higher oviposition rate compared to its quorum sensing (QS) deficient strains [85].

Microbes may also contribute to larval development. They can liberate essential amino acids from carrion resource by digesting the tissues for larval consumption [128]. Microbes acquired horizontally either (e.g., from the growing development) or vertically (from mother to offspring) can assist with metabolization of nutrients or provide direct nutrition such as essential amino acids, that are limited or lacking in the diet due to their limited biosynthetic capabilities [116-118]. Amino acids are important factors for insect nutrition [186]. The essential amino acids, including argine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine, have been demonstrated to be critical for the development and growth of insect [186, 187].

The previous chapter used four VOCs, DMDS (Dimethyl disulfide), indole, PAA (Phenylacetic acid) and isbutylamine, to investigate the effect on adult *L. sericata* behaviors so as to understand the roles of VOCs in carrion resource exploitation by adult flies. The significance of studying these four compounds were discussed in the chapter. Of intrdoduction In general, DMDS, indole and isobutylamine are potential VOCs that are involved in *Proteus* cell-to-cell signaling as well as regulation of blow flies' response [67, 74]. PAA is produced by the commensal bacteria *P. mirablis* [53], and has similarity in structure as well as sharing the same decomposition pathway with phenylethyl alcohol [101], which was also previously determined as one of the potential bacterial QS compound [67, 74]. In the current study, four essential amino acids, methionine,

tryptophan, phenylalanine and valine were used to study the larvae performance on imbalanced dietary as a result of both maternal choice of resource and their own strategies in resource utilization. These four essential amino acids were associated with the previously studied VOCS as described in details in previous chapter. In brief, methionine, tryptophan, phenylalanine and valine are the origins of DMDS [188, 189], indole [104], PAA [115] and isobutylamine [111] individually in microbial degradation. Imbalanced dietary with different essential amino acids compositions were constructed to study larval performance in terms of their food choice and development. Antimicrobials were added into the dietary to study the potential roles of microbes on the performance of larvae on the carrion associated resource. The hypothesis in this study was that the imbalance in dietary amino acids and the presence of microbes will affect larval food preference and development.

Methods

Colony maintenance

L. sericata adults were maintained in 30 x 30 x 30 cm BioQuip bug dorms (Bioquip Products, Rancho Dominguez, CA, USA) [183]. Adult flies were fed bovine liver blood *ad libitum* for the first 7-9 d post emergence in order to stimulate ovarian development. And then were provided with bovine liver in 88.7 ml plastic bath cups (Wal-Mart. Inc., Bentonvillle, AR, USA) for 2-3 d. Larvae of 3-d-old post hatching, were selected and surface cleaned by dipping them in deionized water for 1-2 s and let them dry in filter paper before provided with artificial diet.

Artificial diets

Diets were constructed with different combinations of essential amino acids and other essential nutrients that are of importance to larvae nutrition. Four essential amino acids were chosen for construction of the imbalanced dietary by reducing any of them from the complete diet. The four essential amino acids chosen are phenylalanine (Sigma Aldrich, Basic materials, St. Louis, MO, USA, Purity ≥ 98.0%), methionine (Sigma Aldrich, Basic materials, St. Louis, MO, USA, Purity ≥ 98.0%), tryptophan (Sigma Aldrich, Basic materials, St. Louis, MO, USA, Purity ≥ 98.0%) and valine (Sigma Aldrich, Basic materials, St. Louis, MO, USA, Purity ≥ 98.0%). The diets were treated with or without propionic acid and nipagin, which serves as an antimicrobials and food preservative, and has been documented to inhibit growth of *Proteus* [190, 191]. Stock solutions were all filter sterilized using 0.2 μm syringe filter (cellulose, acetate membrane, VWR® SuperClear™, Radnor, PA, USA). As referred by USDA National Nutrient Database and previous documented studies [98, 192], a modified artificial diet was made in this research as complete diet (Table 8). Amino acids stock solutions were made by dissolving in milliQ water, then diluted to 70% of total volume with dH_2O, and adjusted final pH to 4.5 using HCl or NaOH based on the pH. All the chemicals used in making the diet were all purchased from Sigma (Sigma Aldrich, Basic materials, St. Louis, MO, USA). Restricted diets were made by taking out the essential amino acids from the complete diet, making the diet 1 (D1: complete diet without Phenylalanine), diet 2 (D2: complete diet without methionine), diet 3 (D3: compete diet without valine), diet 4 (complete diet without tryptophan).

Table 8. Constituents and concentrations of complete artificial diets

	Nutrients	Concentration in diet (g/L)
Essential amino acids (EAA)[1]	F(L-Phenylalanine)	3.253
	H (L-Histidine)	1.887
	I (L-Isoleucine)	2.901
	K(L-Lysine)	4.821
	L (L-Leucine)	5.730
	M (L-Methionine)	1.629
	R (L-Arginine)	3.723
	T (L-Threonine)	2.607
	V (L-Valine)	3.780
	W (L-Tryptophan)	0.789
Non-essential Amino Acids (NEAA)	A (L-Alanine)	2.328
	C (L-Cysteine)	0.752
	D (L-Aspartate)	3.854
	E (L-Glutamate)	5.224
	G (Glycine)	2.328
	P (L-Proline)	0.092
	S (L-Serine)	1.810
	Y (L-Tyrosine)	1.614
	Cholesterol[2]	2.750
Trace element solutions[3]	$CaCl_2$	2.720
	$MgSO_4$	9.000
	$CuSO_4$	4.000
	$FeSO_4$	2.430
	$MnCl_2$	9.000
	$ZnSO_4$	1.800
Vitamine[4] and lipid related metabolites[5]	Thiamine	0.002
	Riboflavin	0.027
	Ca- pantothenate	0.071
	Choline-chloride	3.330

[1]Amino acids were all added from 4* stock made in milliQ water; [2] added from10* stock made in ethanol; [3] added from 100* stock made in milliQ water; [4] added from 20 stock made in milliQ water; [5] added from 10* stock made in milliQ water.

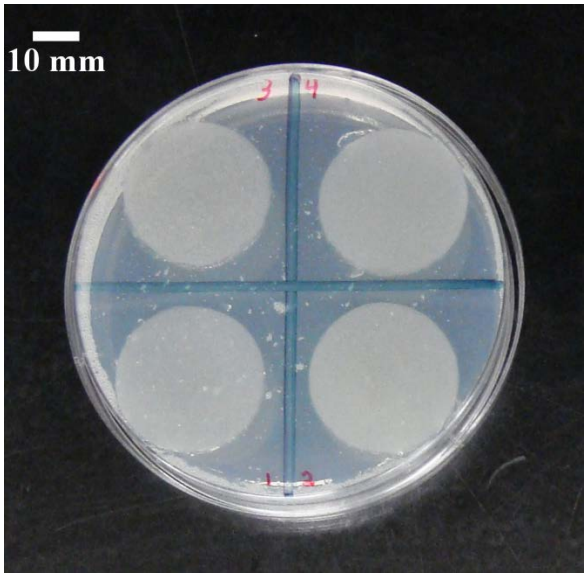


Figure 14. Petridish used for food choice assay. Each petridish was randomly divided into four quadrats, each quadrat would be randomly numbered with number 1 to 4 (radius: 15.5 mm) using a random number generator, and each quadrate will be filled with 4ml of one of the four restricted diet tested. D1: complete diet without Phenylalanine, D2: complete diet without methionine, D3: compete diet without valine, D4 complete diet without tryptophan. All the diets were divided into two groups, where group 1 were diets (D1, D2, D3 and D4) treated with antimicrobials and group 2 (D1, D2, D3 and D4) were diets were not treated with antimicrobials.

Food choice assay

Food choice was evaluated as follows: in each assay trial, six replicates of diets with anti-microbial and another six replicates of the diets without antimicrobials were tested. Diets were prepared in 100*15 mm BD Falcon petri dish (BD Falcon™, Becton, Dickinson and Company, NJ, USA), each of which was randomly divided into four quadrats with number 1 to 4 (radius: 15.5 mm) (Figure 14) using a random number generator, and each quadrate will be filled with one of the four restricted diet tested. All the diets were divided into two groups, where group 1 were diets (D1, D2, D3 and D4)

treated with antimicrobials and group 2 (D1, D2, D3 and D4) were diets without antimicrobials. Three trials were run in total with 18 replicates of petri dish of diets that treated with antimicrobials and another 18 replicates with diets that were without antimicrobials. Twenty larvae of third days old were introduced into the center of the petri dish, and the number of the larvae present on each quadrates was counted continually, twice a day until larvae started to pupate or died.

Development assay

The constructed complete artificial diet were made in reference to the nutrients data from raw beef liver analyzed by USDA National Nutrient Database and previously study [98, 192] and were modified in this current research. The complete diet and the other four diets, each of which deficient in one of the four essential amino acids were adjusted by taking the essential amino acids from the complete diet, were used to investigate the larvae development. The development assay was carried out the same time with the diet choice assay. The larvae of third days old from the same generation in the colony as used in the diet choice assay were used in the assay. The complete diet (D5) and the four deficient diet: diet 1 (D1: complete diet without Phenylalanine), diet 2 (D2: complete diet without methionine), diet 3 (D3: compete diet without valine), diet 4 (complete diet without tryptophan), were prepared and five vials (50 ml Ultra-High Performance Centrifuge Tube, VWR® SuperClear™, Radnor, PA, USA) were set up for each diet. Since all the diets were divided into two groups, group 1 were treated with antimicrobials and group 2 were diets without antimicrobials, 25 vials were used filled

with each kind of diets in each group, resulting 50 vials in one trial. The assay was carried out for three trials in total. Pupal length, width and mass were weighed after assay to see if there was any difference between diet groups (with and without antimicrobials) and diet deficiencies.

Statistical Analysis

The larval diet choice was analyzed with PROC MIX (SAS 2011), with fixed effect of different types of diets, with random effect as days, to analyze the choice of the larvae on different restricted diets with a certain essential amino acid deficiency. The data from development assay was analyzed using PROC GLIMMIX model, with independent variables as whether treated with or without antimicrobials and the different diet types (complete diet, D1, D2, D3 and D4), and dependent variables were length, width and mass of the pupae. A Tukey-Kramer test was used for multiple comparisons of the means in order to determine statistical significance ($P \leq 0.05$). MANOVA (Multivariate Analysis of Variance) was used to test simultaneously if the factors of group, diet types and their interactions have any effect in determining the difference of length, width and mass of pupae. Means were separated using Tukey's least significant difference test (SAS 2011).

Results

Larval diet preferences were evaluated with and without antimicrobials. For diets free of antimicrobials, larvae showed most preference to the diet without methionine

(D2) and least preference to diet without tryptophan (D4) (Figure 15). Using the probability of choosing D4 as a reference, D2 (P = 0.0409) was significantly preferred over D4 (Table 8). Larvae shifted their preferences to D4 when antimicrobials and food preservatives were present in the diets (Figure 16), and they showed least preference to the diet without methionine (D2) (P = 0.0026) and diet without valine (D3) (P = 0.0199) (Table 9). The preference between D2 and D3 was not significant.

Table 9. Solutions of random and fixed effects. Log (Pi/P2) = βi + Radom effect, (i=1, 3, 4; Pi: estimated probability in choosing Di; βi: estimates for fixed effect; day is defined as random effect).

	Effect	Diets	Estimate	Standard error	DF	t value	Pr > |t|	Covariance Parameter Estimates of day
Without antimicrobials	Intercept	D1	0.6125	0.3231	21	1.90	0.071	0.5425
		D2	0.7630	0.3502	21	2.18	0.04	0.7022
		D3	0.6164	0.4891	21	1.26	0.22	1.6210
With antimicrobials	Intercept	D1	-0.1443	0.4385	15	-0.33	0.7466	1.0453
		D2	-1.1417	0.3172	15	-3.60	0.0026	0.4087
		D3	-0.9556	0.3668	15	-2.60	0.0199	0.5969

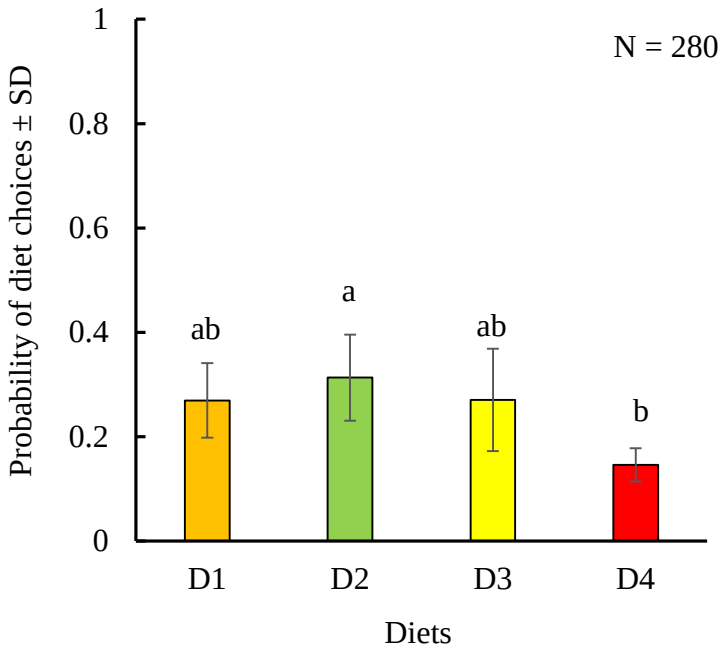


Figure 15. Food choice assay with all diets without antimicrobials**.** D1 to D4 represents the different restricted diet with a certain essential amino acid deficiency, and all the diets were not treated with antimicrobials. Different letters indicate statistically significance ($P < 0.05$). D1: complete diet without phenylalanine, D2: complete diet without methionine, D3: compete diet without valine, D4 complete diet without tryptophan. In the model, D2 was used as reference. Log (P_i/P2) = β_i + Radom effect, (i=1, 3, 4; P_i: estimated probability in choosing D_i; β_i: estimates for fixed effect; day is defined as random effect).

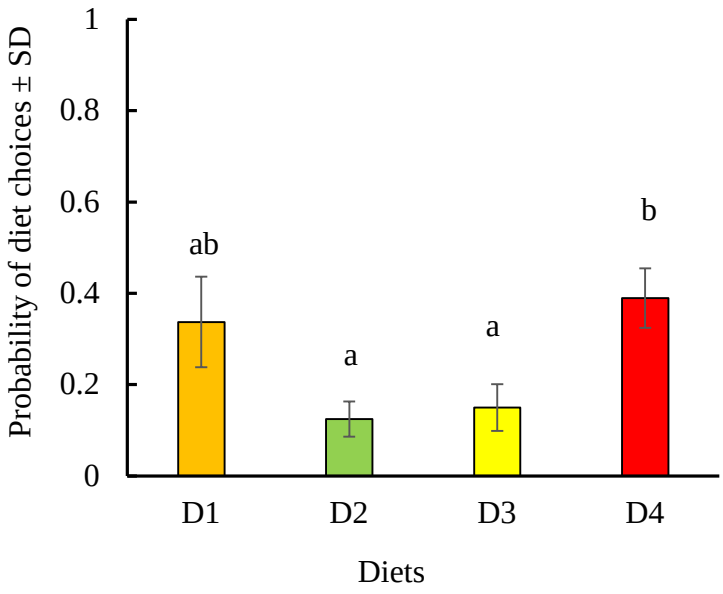


Figure 16. Food choice assay with all diets with antimicrobials. D1 to D4 represents the different restricted diet with a certain essential amino acid deficiency, all the diets were treated with antimicrobials. Different letters above the columns indicated there were significant difference between the probability of choice of D2 and D4, and D3 and D4 ($P \leq 0.05$).D1: complete diet without Phenylalanine, D2: complete diet without methionine, D3: compete diet without valine, D4 complete diet without tryptophan. In the model, D2 was used as reference. Log (P_i/P2) = β_i + Radom effect, (i=1, 3, 4; P_i: estimated probability in choosing D_i; β_i: estimates for fixed effect; day is defined as random effect).

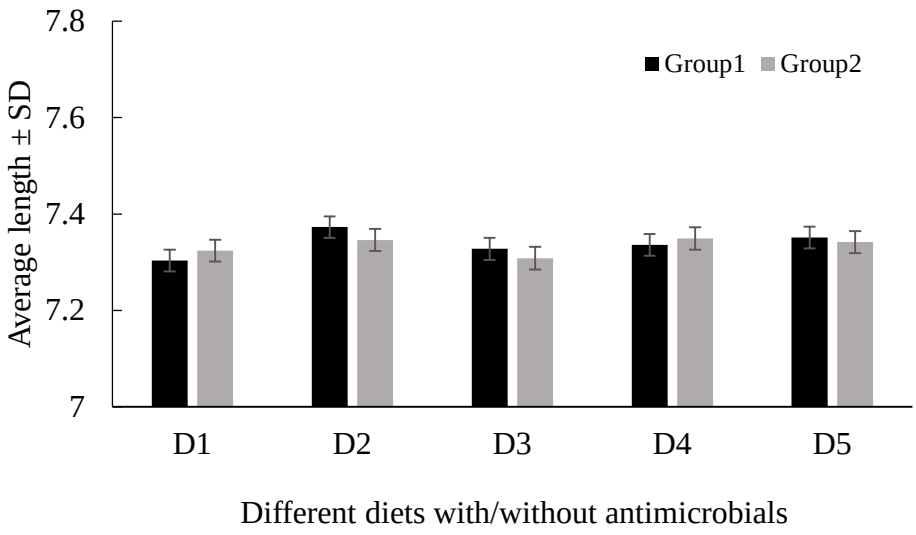


Figure 17. Average length of pupae. There was no significant difference between diets treated and without antimicrobials within each different diet types ($F_{9,736} = 0.85$, $P = 0.5694$). D1: complete diet without Phenylalanine, D2: complete diet without methionine, D3: compete diet without valine, D4: complete diet without tryptophan, D5: complete diet with all the essential amino acids. Group 1: diets treated with antimicrobials, Group2: diets without antimicrobials.

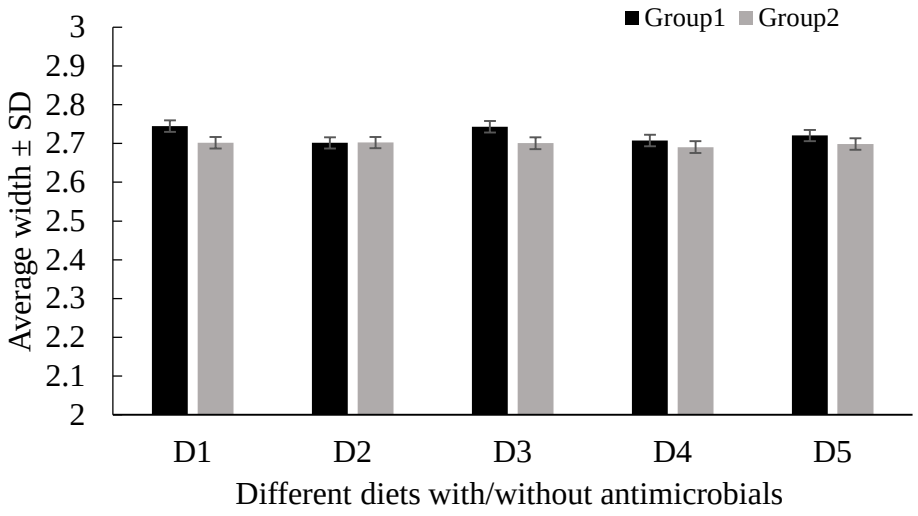


Figure 18. Average width of pupae. There was no significant difference between diets treated and without antimicrobials within each different diet types ($F_{9,736} = 0.94$, $P = 0.4864$). D1: complete diet without Phenylalanine, D2: complete diet without methionine, D3: compete diet without valine, D4: complete diet without tryptophan, D5: complete diet with all the essential amino acids. Group 1: diets treated with antimicrobials, Group2: diets without antimicrobials.

As for the development assay, larval performance was also evaluated in the presence and absence of antimicrobials. None of the factors evaluated differed across the diets nor did they change with antimicrobial treatments. Pupal width, length and mass were measured. There was no significant difference in average length between diets treated and without antimicrobials within each different diet types ($F_{9,736} = 0.85$, $P = 0.5694$) (Figure 17). There was no significant difference in the average width between diets treated and without antimicrobials within each different diet types ($F_{9,736} = 0.94$, $P = 0.4864$) (Figure 18). There was no significant difference in average mass between diets

treated and without antimicrobials within each different diet types ($F_{9,736} = 0.58$, $P = 0.8169$) (Figure 19).

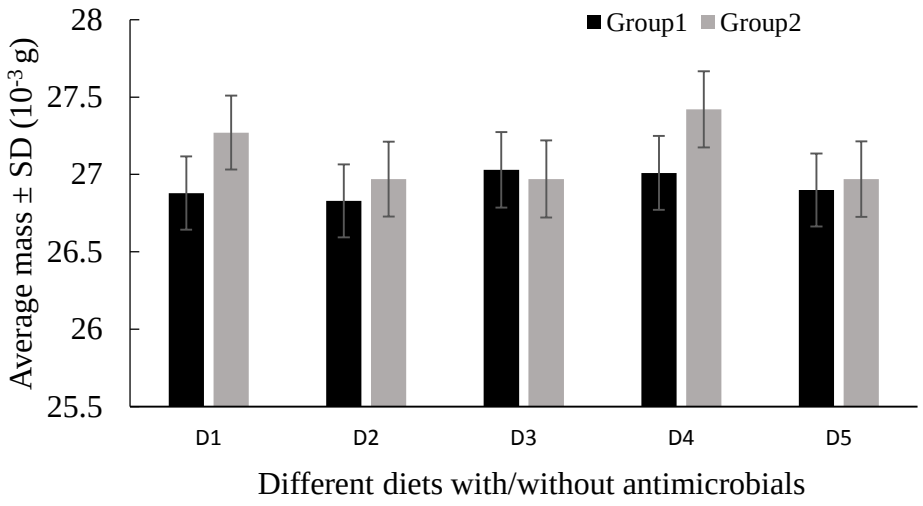


Figure 19. Average mass of pupae. There was no significant difference between diets treated and without antimicrobials within each different diet types ($F_{9,736}=0.58$, $P = 0.8169$). D1: complete diet without Phenylalanine, D2: complete diet without methionine, D3: compete diet without valine, D4: complete diet without tryptophan, D5: complete diet with all the essential amino acids. Group 1: diets treated with antimicrobials, Group2: diets without antimicrobials.

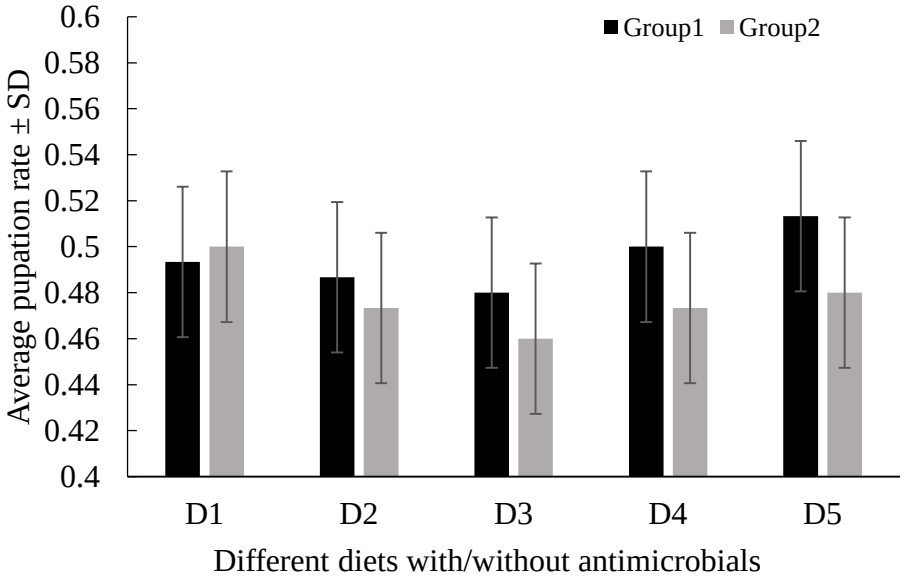


Figure 20. Average pupation in development assay. Average pupation rate were measured. There was no significant difference between diets treated and without antimicrobials within each different diet types ($F_{9,149} = 0.22$, $P = 0.9988$). D1: complete diet without Phenylalanine, D2: complete diet without methionine, D3: compete diet without valine, D4: complete diet without tryptophan, D5: complete diet with all the essential amino acids. Group 1: diets treated with antimicrobials, Group2: diets without antimicrobials**.**

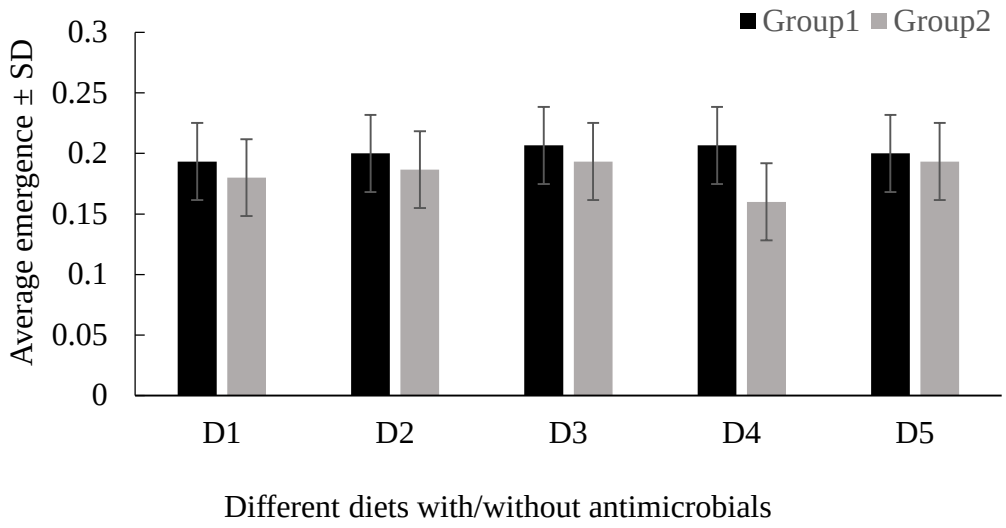


Figure 21. Average emergence in development assay. Average emergence rate of pupae were measured. There was no significant difference between diets treated and without antimicrobials within each different diet types ($F_{9,149} = 0.19$, $P = 0.9946$). D1: complete diet without phenylalanine, D2: complete diet without methionine, D3: compete diet without valine, D4: complete diet without tryptophan, D5: complete diet with all the essential amino acids. Group 1: diets treated with antimicrobials, Group2: diets without antimicrobials.

The pupation rate was not significantly different between diets treated and without antimicrobials within each different imbalanced dietary ($F_{9,736} = 0.94$, $P = 0.4864$) (Figure 20). The emergence rate was not significantly different between diets treated and without antimicrobials within each different diet types ($F_{9,149} = 0.19$, $P = 0.9946$) (Figure 21). The emergence rate seemed to be slightly higher in complete diet compared to other deficient diet treated with antimicrobials, however the difference was not significant.

Discussion

In previous chapter, the effects of VOCs on adult behaviors were demonstrated. Based on the VOCs and related essential amino acids that are associated with carrion decomposition, diets that were imbalanced in essential amino acids were constructed in the current study and the effect of maternal choice on larval performance in terms of their diet preferences and development were tested and demonstrated in this chapter. The results showed that food preferences were significantly different toward different imbalanced diets and preferences were significantly affected by the addition of antimicrobials in the diets. When given the choices of four different diet that are deficient in one of the essential amino acids, *L. sericata* showed most preference to diet without methionine, and least preference to diet without tryptophan. When antimicrobials were added into all the diets, larvae showed avoidance to diet without methionine. However, none of the essential amino acids deficiency or antimicrobial treatment significantly affect the development of larvae in the current study.

The antimicrobials used in the current study has been documented to inhibit growth of *Proteus* [190, 191]. By showing the feeding patterns of larvae feed on the diets treated with and without antimicrobials may elucidate the role of *Proteus* in larval nutrition. As documented before, *P. mirabilis* has been recurrently isolated from *L. sericata* larval gut [67]. The pathway of biosynthesis of methionine, has been studied in *P. mirabilis* [95], Since *Proteus* contributes to the production of methionine, it is possible that with the presence of antimicrobials that inhibit *Proteus*, larvae may need to

increase their intake from the diets with methionine and decreased the visits to the diet without methionine.

On the other hand, the commensal bacterium *Proteus* can produce antibacterial agents “mirabilicides” like PAA, which is capable of killing microbes that are present in the fly larvae [193]. Thus it is also possible that the presence of *Proteus* in *L. sericata* larvae has an effect on the compositions of the larval microbiota. The addition of antimicrobials might affect the gut microbial composition by changing the amount of PAA produced by the presence/reduced levels/absence of *Proteus*, thus adds up to their shifted feeding behaviors observed on diets treated with antimicrobials. Methionine is also known to enhance swarming motility of *Proteus* strains [95, 97] and was suggested its potential role in *Proteus* swarming differentiation as being the precursor of QS molecules [190]. Thus, performance of *Proteus* in the absence of methionine could also be a contributing factor. In the current study, when all the diets were treated with antimicrobials, larvae avoid diet without methionine when all diets were treated with antimicrobials. It is possible that the addition of antimicrobials reduced *Proteus* density thus resulting in reduced production of QS molecules. Larvae prefers to diet with methionine so that its commensal bacteria *Proteus* can use it to “rescue” their QS behaviors and to resume the *Proteus* density as well as the gut microbial compositions inside the larvae.

Different aspects of performance may reflect different nutrition requirement [194], Thus, the criterion used in the development assay in the current study might not

be adequate enough to indicate the role of the essential amino acids in larval performance.

It is also worth noting that all the larvae teste in the current study were three days old. This was done as that was the easiest stage to keep alive, without escapes from the containers used. It will be necessary to test younger larvae in further study so as to construct a stronger relation between maternal choice and larval performance. This is especially important as younger larvae are more likely to be impacted by suboptimal diets.

Adult and larval behaviors are connected. Adult decisions translate into larval preferences due to their abilities, or inabilities, to perform well on maternally chosen resources. The association between adult resource preference and larval performance is poorly understand in carrion feeding flies. My current investigation of larval performance on the diets deficient in amino acids that provide VOC cues to maternal flies may provide a stepping stone for understanding the strategies of larvae in ephemeral and patchy resource utilization when faced with limited choices in their living environment. Further research into the gut microbial fauna compositions involved in the larval performance such as food preferences could reveal the nutritional roles of symbionts in blow fly-microbes interactions.

CHAPTER V

EFFECTS OF VOLATILE ORGANIC COMPOUNDS ON LIFE HISTORY TRAITS OF LUCILIA SERICATA (MEIGEN) (DIPTERA: CALLIPHORIDAE)

Introduction

Insects sense environmental cues through their perceptual systems to obtain the information they need regarding food, mates, kin and predators [67]. Obtained environmental information allows the insect to assess the current state of their surroundings and adjust development, behaviors, and physiology accordingly [195-200]. Specific sensory cues such as yeast odor, and the neurosensory and neuroendocrine circuits associated with an insect are involved in modulating conserved mechanisms of physiology as related to the aging process of insect [154, 201, 202].

Detection of compounds indicating the presence of a mate contribute to the metabolic regulation of reproduction by the recipient [203]. Although not insect-based, crude pheromone extracts have also been reported to impact nematode lifespan [204]; however the active component is still in question [204-206]. Perception of dauer pheromone, which reflects population density [195], or a compound that originates from organic material and reflects food availability [207, 208], may regulate the lifespan of the nematode *Caenorhabditis elegans* [208]. Similarly, in *C. elegans*, life extension can be achieved through dietary restriction by bacterial food deprivation; however, some diffusible components produced by bacteria were indicated to partially suppress the life

extension, by which the survival of *C. elegans* life was decreased compared to *C. elegans* maintained on agar plates with no bacteria present [209].

The connection between sensing and longevity has been also demonstrated for insects [150, 154]. Exposure to nutrient odorants (e.g. yeast odorant) derived from microbial processes [150-152] can modulate life span and partially reverse the longevity-extending effects of dietary restriction on *Drosophila*, in which flies fed with restricted diets and exposed to yeast odor showed reduced life span than flies treated with restricted diets without perception of yeast odor [150]. Sensory perception of CO_2 may provide information to *D. melanogaster* on the availability and quality of food sources [206]. Loss-of-function mutation in odorant receptor Gr63a increased life span up to 30% more than what was observed for their siblings with functional receptors [154]. These results showed the modulation of life span through the detection of odor by an olfactory neuron [150, 208, 210], which were indicated to be involved in insulin-like growth factor signaling [150, 208, 210-212], thus exposure to environmental cues may affect insect aging through altered olfaction response associated with insulin signaling and subsequent modulation of transcription factors [150].

Defecation responses are relatively unstudied, but potentially have profound repercussions on survival, success of offspring [213], and the ability of insects to spread disease [214]. Water loss from desiccation may senescence. Microbes transferred from the parent to larval resources may affect the survival or relative success of immatures. Finally, defecation is a mechanism by which pathogenic microbes can be disseminated throughout the environment. Defecation ratio in *Drosophila* was found to be related to

physical stimulation and genotypes [215]. Wild type flies that were disturbed had higher defecation ratio compared to undisturbed mutant flies [215]. However the implications of insect defecation as related to life-history traits in response to perception of environmental cues such as resource associated volatile organic compound (VOCs) are not known.

After death, animals are decomposed by a variety of microorganisms, the decaying process is usually associated with releasing of assorted VOCs [67, 158]. VOCs produced from microorganisms signal arthropods to the presence of resources such as food, host, appropriate oviposition sites and mates [158]. Decomposing remains are ephemeral resources, which last only a few days to a few weeks. Thus colonizers have to possess efficient resource exploitation strategies so as to be effective competitors [149]. *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) is considered a primary colonizer of carrion [149]. They use carrion as resource for food, mating and oviposition [67]. Because of such a high level of dependence on carrion, they have highly sensitive olfactory systems enabling them to detect low levels of VOCs indicative of the presence of these resources [180-182]. VOCs released from the decomposing remains can serve as cues for the regulation of attraction and/or repellence of blow flies [129-132]. In an attempt to study the possible interkingdom relation between *L. sericata* and bacteria, a commensal bacterium *Proteus mirabilis* was isolated from *L. sericata* salivary gland, and a series of mutants with reduced swarming ability were also constructed [67]. Wild type *Proteus* attracted more adult flies and resulted in higher chances of oviposition compared to one of its mutant [67]. Further study demonstrated the VOCs produced by

the *Proteus* wild type and mutant differed [74]. The differences in composition of VOCs may explain the variation in attraction and oviposition rates by blow flies observed [74].

DMDS (DMDS) was one of the key VOCs that were differentially regulated between the wild type and swarming deficient *Proteus,* in that DMDS collected from wild type showed relatively higher concentration and was still under detection after 7 hours [74]. Sulfur compounds, such as DMDS, are prominent VOCs emitted by decaying vertebrates [216]. Emission of volatile sulphur compounds, such as hydrogen sulfide, DMDS, dimethyl trisulfide, dimethyl tetrasulfide and methionol, are the result of the microbial degradation of sulphur-containing essential amino acids such as cystein and especially methionine [93, 189, 217]. *P. mirabilis* possesses enzymes allowing it to break down methionine [86, 92], the process of which is composed of methionine, deamination, demethiolation and oxidation of before the formation of DMDS [218].

In chapter III, I tested *L. sericata* response to different DMDS doses and found that flies of different sexes and ovarian status showed different response. The objective of my study in this chapter was to determine the effect of detecting DMDS, an indicator of the presence of carrion, on life-history related traits, such as life span and defecation, of *L. sericata.* I used DMDS as a model volatile compound since it is associated with carrion resource, *L. sericata* and its commensal bacteria *Proteus* as described above. Here, I hypothesize that there is a connection between detecting VOCs and physiological responses by *L. sericata*.

Methods

Colony maintenance

L. sericata adults were maintained in 30 x 30 x 30 cm BioQuip bug dorms (BioQuip Inc., CA, USA) [183]. Adult flies were provided with bovine liver blood *ad libitum* in 88.7 ml plastic bath cups (Walmart Inc., Bentonvillle, AR, USA) for 7-9 d post emergence in order to stimulate ovarian development.

Resulting eggs and liver were placed into 900 ml mason jars. The bottom 5 cm of the jars was filled with sawdust. Larvae dispersing from the liver were allowed to pupate in the saw dust. Pupae used for this research were then transferred into a different 27°C and a 12:12 L: D cycle in a walk-in growth chamber and were fed with granulated sugar and water *ad libitum* post emergence. Flies of age 3-5 days old, 7-9 days old, 11-14 days old were used later for bioassay, and were grouped as age1, age2, and age3 respectively.

Exposure to DMDS

DMDS (Sigma Aldrich, St. Louis, Missouri, USA, Purity ≥ 99.0%) was diluted with deionized water to concentrations of (5 μg/ml, 0.5 μg/ml, and 0.05 μg/ml. Approximately 10 mL of each tested solution was put into 100 mm × 15 mm glass petri dish (Sigma Aldrich, St. Louis, MO, USA) with 10mL of water was used for control. Either a treatment or control was placed in the petri dish, which was then placed in a 21 x 12 cm mosquito breeder (BioQuip Inc., CA, USA). Approximately 12 female and 12 male flies were placed into the top of one Funnel Trap where the bottom is the placed with one petri dish filled with either treatment solution or control. Over the top of the

trap, a cotton ball (The Kroger Co., Cincinnati, OH, USA) soaked with sugar and deionized water was place above the screen to keep flies from hungry and dehydration. The total 24 flies were exposed to volatile emission from treatment chemical or control for 24 hours, before recording defecation and life span for each individual fly. Exposure to DMDS was conducted at 20ºC -23ºC and on a 12:12 L: D photoperiod.

Bioassay for defecation and life span

Post exposure to chemicals or control, each fly was transferred into a single petridish (100*15mm BD Falcon™ petri dish (Dickinson and Company, NJ, USA) individually. Inside each petri dish, a filter paper in the shape of circular sector (θ = 22.5°, radius 4.5 cm) (Fisher Scientific, Waltham MA, USA) soaked with deionized water was placed at the bottom to prevent fly from dehydration. The number of defecation spots were recorded each day and then each fly would be transferred to a new petri dish with a piece of water soaked filter paper inside (θ = 22.5°, radius 4.5 cm). The same process was repeated each day until fly died, then the remainder days of each fly post exposure were also recorded. In the current study, the "life span" indicates the remaining days of each fly post exposure to either treatment or control. All the flies were divided into three groups based on their age range.

Data analysis

The defecation data was analyzed with PROC GLMMIX (SAS 2011) full factorial design, using a full factorial design. The model considered the fixed factors of

Sex (means fly types: based on their sexes), AG (means different age groups) and Test (means the exposure to DMDS treatments or control), as well as the 2-way and 3-way interactions of these factors. Least square means were used to compare the differences among groups. The results from analysis were first sorted by day to show defecation on each day, and then pulled all the data together to show the change of defecation through the lengths of days. The life span data also were analyzed with PROC GLMMIX (SAS 2011) full factorial design, in the model of which the fixed factors included the types of flies, different age groups and exposure to different concentrations of DMDS solution, and the interactions of these factors, Tukey's test was used to compared the difference of lease squares means ($P \leq 0.05$). These analysis sought to answer the questions: 1) whether exposure to different concentrations result in different defecation and/ or remaining life expectancy (the remaining living days post exposure to different DMDS concentrations) 2) whether females and males show different defecation and/ or aging after exposure to different treatment 3) whether flies show different defecation at different ages 4) whether there is any interaction effect between any factors of age, sexes and concentrations that affect the life history related traits of flies as mentioned above after exposure to different treatments.

Results

The effects of exposure to different DMDS concentrations on the life history related traits of *L. sericata* were tested. The number of defecation spots and remaining life expectancy of each fly were recorded each day after exposure to different DMDS

concentrations. The model used in the statistical analysis with 3-way interactions of Sex*AG*Tests was a slight improvement over the model without this term. The AIC (Akaike information criterion) is smaller in the model with the 3-way interaction included versus the model without this term (Table 10).

Table 10. Fit Statistics of models for testing the effect of VOCs on life history related traits.

	Model without 3-way interactions	Model with 3-way interactions
AIC (Akaike information criterion)	11022.72	10902.19
AICC (small-sample-size corrected version of Akaike information criterion)	11023.26	10903.13
BIC (Bayesian information criterion)	11115.78	11026.27

Table 11. Significant differences of Least Squares Means of defecations between different combinations of sex, DMDS concentrations, age groups on day 1 post exposure. (Adjustment P value for Multiple Comparisons: Tukey-Kramer).

Sex	Tests	AG	vs	Sex	Test	AG	Estimate	SE	DF	t Value	Adj P
female	0.5	3	vs	male	0.5	3	1.48	0.1	0.12	5.02	0.0002
female	5	3	vs	female	control	3	1.26	0.1	0.06	4.78	0.0005
male	0.05	1	vs	male	0.05	2	1.579	0.1	0.16	4.51	0.0018
female	5	2	vs	male	5	2	0.66	0.1	0.06	-4.35	0.0034
male	0.05	2	vs	male	0.05	3	1.51	0.1	0.15	4.3	0.0043
female	0.05	1	vs	female	0.05	2	1.44	0.1	0.13	4.03	0.0009
male	5	2	vs	male	5	3	1.44	0.1	0.13	3.95	0.0169
female	0.5	1	vs	male	0.5	1	1.36	0.1	0.11	3.91	0.0201
male	0.5	1	vs	male	0.5	3	1.42	0.1	0.13	3.9	0.0209
male	0.5	1	vs	male	control	1	0.70	0.1	0.05	-3.85	0.0245
female	0.5	3	vs	female	5	3	0.80	0.1	0.05	-3.79	0.0309
male	0.05	3	vs	male	0.5	3	0.70	0.1	0.07	-3.70	0.0416

Table 12. Significant differences of Least Squares Means of defecations between different combinations of sex, DMDS concentrations, age groups on day 2 post exposure. (Adjustment *P* value for Multiple Comparisons: Tukey-Kramer).

Sex	Tests	AG	vs	Sex	Test	AG	Estimate	SE	DF	t Value	Adj *P*
male	0.05	1	vs	male	0.05	3	2.33	1.1	735	5.09	0.0001
female	0.05	2	vs	female	5	2	1.68	1.1	735	4.99	0.0002
female	0.05	3	vs	male	0.5	3	2.23	1.2	735	4.76	0.0006
female	control	1	vs	female	control	3	1.37	1.1	735	4.68	0.0008
male	5	2	vs	male	5	3	2.12	1.2	735	4.51	0.0018
female	0.5	3	vs	male	0.5	3	2.03	1.2	735	4.33	0.0039
male	0.05	1	vs	male	0.05	2	2.12	1.2	735	4.31	0.0042
female	5	2	vs	female	control	2	0.67	1.1	735	-4.21	0.0063
male	0.05	2	vs	male	5	2	0.51	1.2	735	-3.88	0.0228
female	0.05	1	vs	female	0.05	2	0.69	1.1	735	-3.84	0.0265
male	5	1	vs	male	5	2	1.44	1.1	735	3.5	0.0807
female	0.05	1	vs	female	0.5	1	0.50	1.1	735	-7.66	<.0001
female	0.05	2	vs	female	0.05	3	1.64	1.1	735	5.57	<.0001
female	0.05	2	vs	male	0.05	2	3.14	1.2	735	7.46	<.0001
female	0.5	1	vs	female	0.5	2	1.98	1.1	735	7.13	<.0001
female	0.5	1	vs	female	0.5	3	2.47	1.1	735	10.01	<.0001
female	0.5	1	vs	female	5	1	1.81	1.1	735	6.8	<.0001
female	0.5	1	vs	female	control	1	1.52	1.1	735	6.38	<.0001
female	0.5	1	vs	male	0.5	1	1.68	1.1	735	5.2	<.0001
female	5	3	vs	male	5	3	2.43	1.2	735	6.01	<.0001
female	control	2	vs	male	control	2	2.09	1.1	735	10	<.0001
female	control	3	vs	male	control	3	2.21	1.1	735	8.21	<.0001
male	0.5	1	vs	male	0.5	3	2.98	1.2	735	6.08	<.0001
male	5	1	vs	male	5	3	3.05	1.2	735	7.02	<.0001
male	control	1	vs	male	control	2	1.90	1.1	735	7.5	<.0001
male	control	1	vs	male	control	3	2.69	1.1	735	8.89	<.0001

Defecation

The type 3 tests of fixed effects on fly defecation were demonstrated based on the number of defecation spots recorded on each day post exposure of different DMDS concentrations (Appendix A-2).

On day one post exposure, sex significantly explained the differences in the number of defecation spots per fly ($F_{1,\ 2}$ = 282.9, df_1 = 1, df_2 = 1269, P <0.0001) (Appendix A-2). The number of defecation spots were higher in females than males (Figure 22). Exposure to different DMDS concentrations significantly explained the differences in the number of defecation spots per fly ($F_{1,\ 2}$ = 2.92, df_1 = 3, df_2 = 1269, P = 0.0331) (Appendix A-2). Fly age was also significant in explaining the differences in the number of defecation spots per fly ($F_{1,\ 2}$ = 100.53, df1 = 2, df2 = 1269, P < 0.0001). The number of defecation spots were compared among flies of different sex, age groups and the DMDS concentrations treatments on day 1 post exposure, and the comparisons with significant difference in number of defecation spots were demonstrated in Table 11.

On day two post exposure, sex significantly explained the difference in the number of defecation spots per fly ($F_{1,\ 2}$ = 139.01, df_1 = 1, df_2 = 735, P < 0.0001) (Appendix A-2). The number of defecation spots were higher in females than males (Table 12, Figure 22). Exposure to different DMDS concentrations significantly explained the differences in the number of defecation spots per fly ($F_{1,\ 2}$ = 7.77, df_1 = 3, df_2 = 735, P = 0.0331) (Appendix A-2), age was also significant in explaining the differences in the number of defecation spots per fly ($F_{1,\ 2}$ = 41.19, df_1 = 2, df_2 = 735, P < 0.0001) (Appendix A-2). The number of defecation spots were compared among flies of

different sex, age groups and the DMDS concentrations treatments on day 2 post exposure, and the comparisons with significant difference in number of defecation spots were demonstrated in Table 11.

On day three post exposure, sex significantly explained the difference in the number of defecation spots per fly ($F_{1,\ 2}$ = 9.54, df_1 = 1, df_2 = 323, P = 0.0022) (Appendix A-2). The number of defecation spots were higher in females than males (Figure 22). Exposure to different DMDS concentrations significantly explained the differences in the number of defecation spots per fly ($F_{1,\ 2}$ = 3.26, df_1 = 3, df_2 = 323, P = 0.0219) (Appendix A-2), age was also significant in explaining the differences in the number of defecation spots per fly ($F_{1,\ 2}$ = 8, df_1 = 2, df_2 = 323, P = 0.0004) (Appendix A-2). The number of defecation spots were compared among flies of different sex, age groups and the DMDS concentrations treatments on day 3 post exposure, and the comparisons with significant difference in number of defecation spots were demonstrated in Table 13.

Table 13. Significant differences of Least Squares Means of defecations between different combinations of sex, concentrations, age groups on day 3 post exposure. Adjustment *P* value for Multiple Comparisons: Tukey-Kramer.

Sex	Tests	AG	vs	Sex	Tests	AG	Estimate	SE	DF	t Value	Adj *P*
male	0.5	2	vs	male	5	2	0.36	1.30	323	-3.87	0.0255
female	0.05	2	vs	female	0.5	2	2.08	1.21	323	3.81	0.0317
male	5	2	vs	male	control	2	1.83	1.18	323	3.73	0.0407

female	0.05	3	vs	male	0.05	3	3.12	1.36	323	3.7	0.0446
female	control	1	vs	female	control	2	1.53	1.13	323	3.51	0.0826
female	0.05	2	vs	female	control	2	2.07	1.12	323	6.3	<.0001

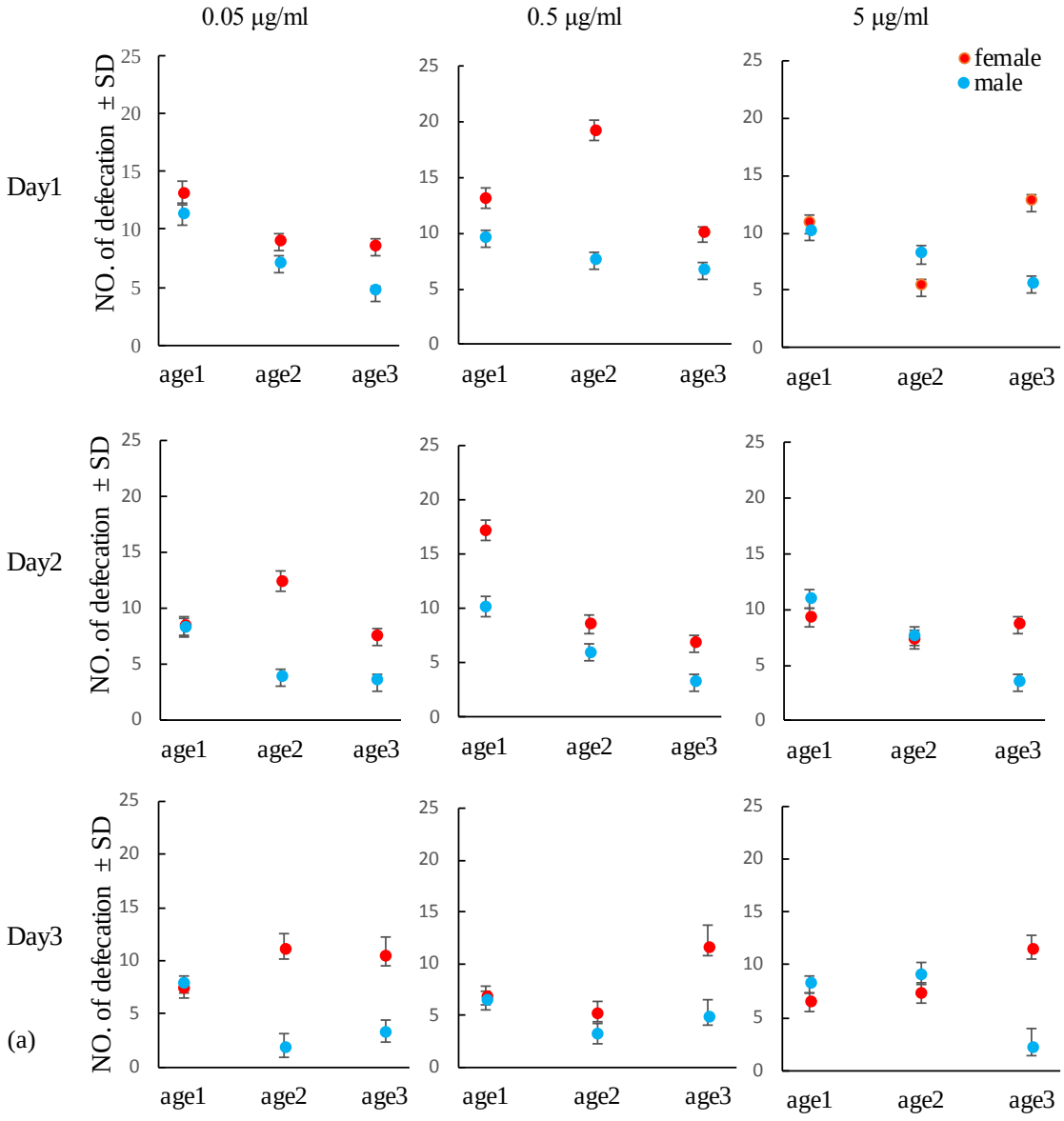


Figure 22. Number of defecation spots of flies at different age groups, different sexes on day1-3 after exposure to DMDS at 0.05 μg/ml, 0.5 μg/ml and 5 μg/ml. Age1 indicates flies of age 3-5 days old, age2 indicates flies of 7-9 days old, and age 3 indicates flies of 11-14 days old. Age1: age grou1, age 2: age group2, age3: age group3.

On day four post exposure, sex significantly explained the differences in the number of defecation spots per fly ($F_{1, 2} = 5.4$, $df_1 = 1$, $df_2 = 139$, $P = 0.0215$) (Appendix A-2), exposure to different DMDS concentrations marginally correlated with defecation rates ($F_{1, 2} = 2.36$, $df_1 = 3$, $df_2 = 139$, $P = 0.0737$) (Appendix A-2), age was significant in explaining the differences in the number of defecation spots per fly ($F_{1, 2} = 14.06$, $df_1 = 2$, $df_2 = 139$, $P < 0.0001$) (Appendix A-2). The number of defecation spots per fly was generally lower than in previous three days (Figure 23).

On day five post exposure, sex did not significantly explain the differences in the number of defecation spots per fly ($F_{1, 2} = 0.16$, $df_1 = 1$, $df_2 = 65$, $P = 0.6919$) (Appendix A-2); however exposure to different concentrations still significantly explained the differences in the number of defecation spots per fly ($F_{1, 2} = 4.23$, $df_1 = 3$, $df_2 = 65$, $P = 0.0086$) (Appendix A-2), age was not significant in explaining the differences in the number of defecation spots per fly ($F_{1, 2} = 2.08$, $df_1 = 1$, $df_2 = 65$, $P < 0.0001$) (Appendix A-2).

On day six post exposure, sex significantly explained the differences in the number of defecation spots per fly ($F_{1, 25} = 4.44$, $df_1 = 1$, $df_2 = 25$, $P = 0.0452$) (Appendix A-2). However exposure to different DMDS concentrations did not significantly explain the difference in the number of defecation spots per fly ($F_{1, 2} = 1.8$, $df_1 = 2$, $df_2 = 25$, $P = 0.1855$) (Appendix A-2). No other results were generated for this age group due to insufficient data.

On day seven post exposure, no result generated as for the effect of sex, exposure to different DMDS concentrations and age, since there were insufficient data available on day 7 due to only a few flies survived on day 7.

Through the length of all the days, sex was significant in explaining the differences in the number of defecation spots per fly ($F_{1, 2} = 167.37$, $df_1 = 1$, $df_2 = 2019$, $\alpha=0.05$, $P < 0.0001$) (Appendix A-2), age significantly explained the difference in the number of defecation spots per fly as well ($F = 56.4$, $df_1 = 2$, $df_2 = 2019$, $\alpha=0.05$, $P < 0.0001$) (Appendix A-2).

Over the length of the experiment, females had significant higher number of defecation spots per fly compared to males when exposed to DMDS (t value=10.53, df = 2019, $\alpha = 0.05$, $P < 0.0001$) (Appendix A-4). Flies 3-5-day-old had significantly higher number of defecation spots when exposed to DMDS than the other fly age groups (Appendix A-4). Flies age 11-14 days old had the lowest defecation rate within the tested age groups (Appendix A-4 and Figure 24). Female flies exposed to DMDS at concentration of 0.5 μg/ml had higher defecation rates compared to exposure to other concentrations at 0.05 μg/ml and 5 μg/ml (Figure 24). Female flies at age 7-9 days old had significantly less amount of defecation spots post exposure to DMDS at 5 μg/ml, compared to females of the same age that were exposed to DMDS at 0.05 μg/ml (df = 2019, t value = -4. 08, $\alpha=0.05$, $P =0.023$), 0.5 μg/ml (df = 2019, t value = -5.01, $\alpha=0.05$, $P < 0.0001$), and control (df = 2019, t value = -3.86, $\alpha=0.05$, $P =0.023$) (Figure 24 and Appendix A-4).

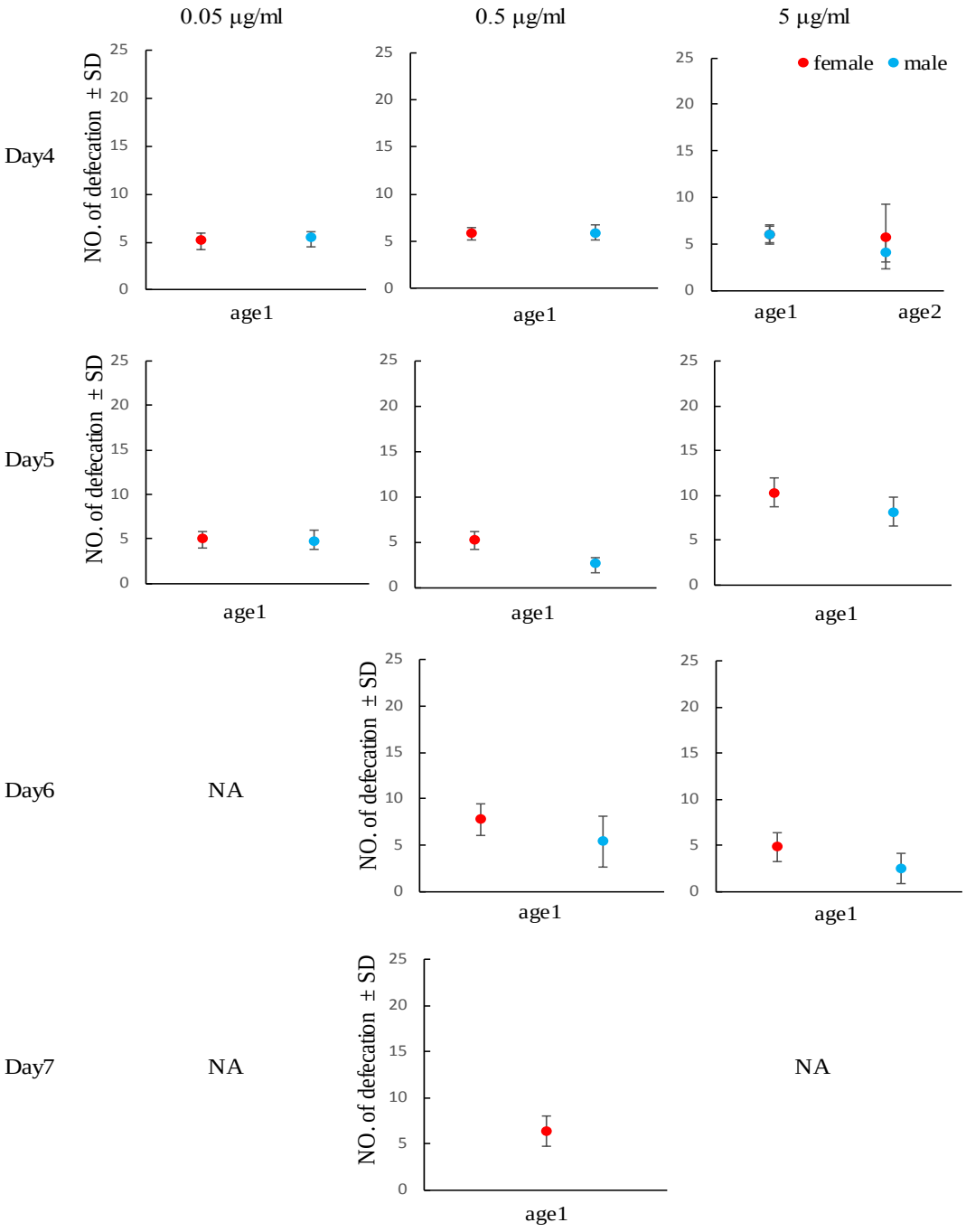


Figure 23. Estimated mean of numbers of defecation spots of flies at different age groups, different sexes on day 4-7, after exposure to DMDS at 0.05 µg/ml, 0.5 µg/ml and 5 µg/ml. The NA in day 6 and day 7 indicated no result generated due to insufficient data input since there were only a few flies left and very limited amount of defecation by them. Age1 indicates flies of age 3-5 days old, age2 indicates flies of 7-9 days old, and age 3 indicates flies of 11-14 days old.

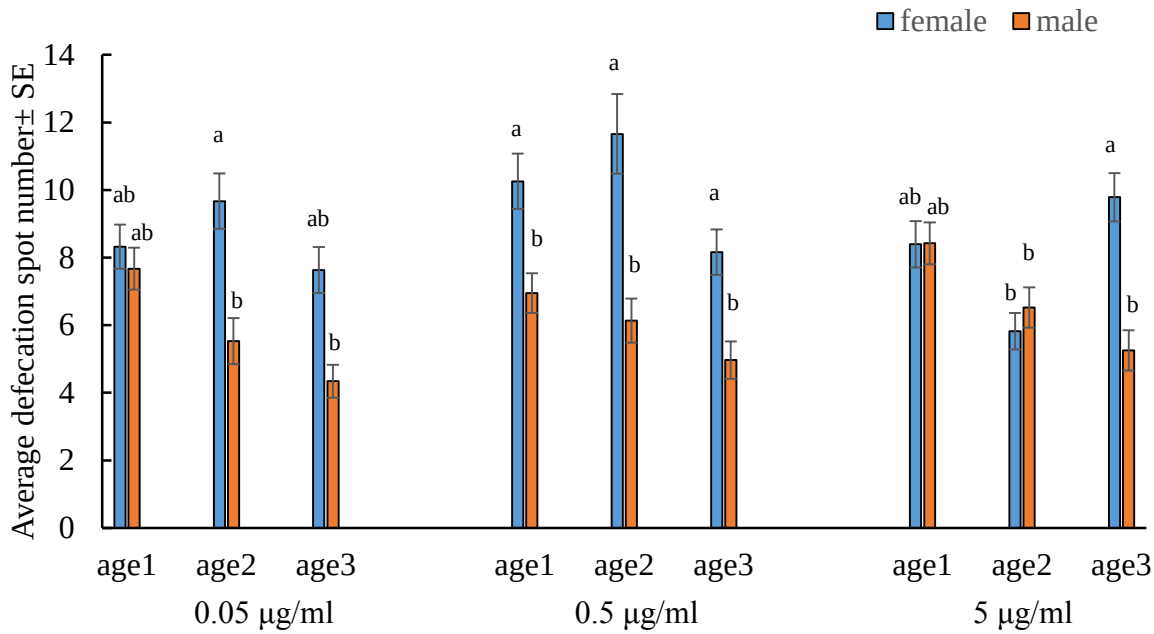


Figure 24. Average defecation spots number ± SE by flies of different age groups, different sexes post exposure to DMDS at different concentrations. Age1 indicates flies of age 3-5 days old, age2 indicates flies of 7-9 days old, and age 3 indicates flies of 11-14 days old.

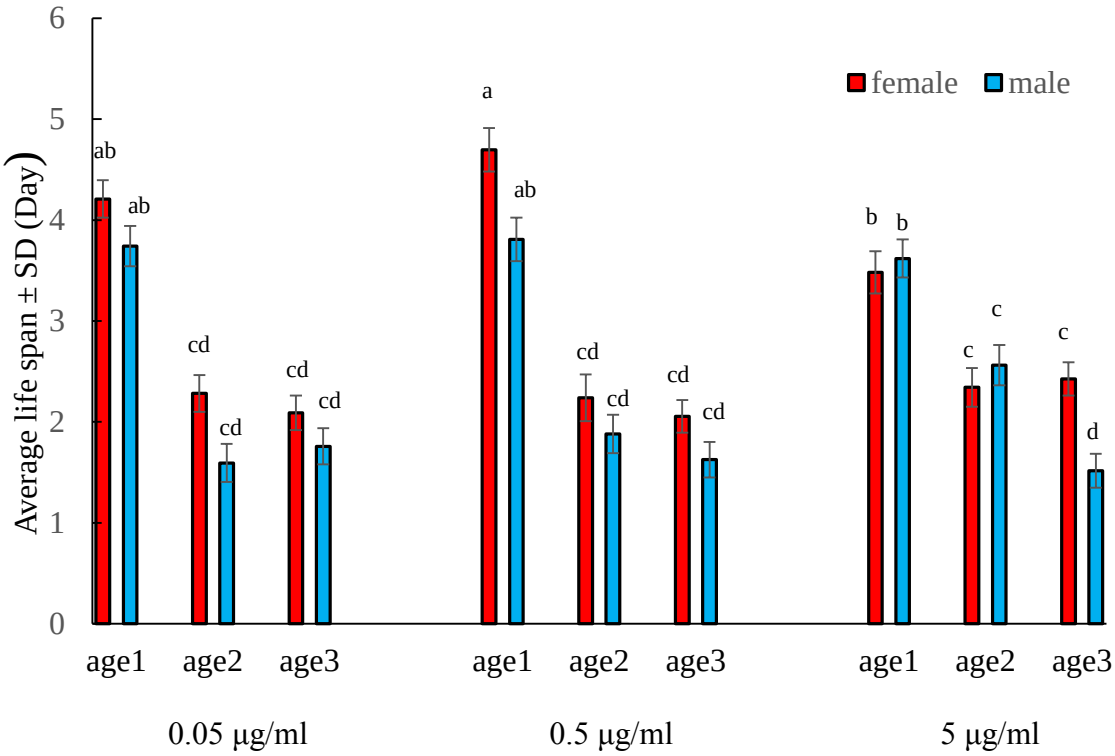


Figure 25. Average life span ± SE of flies of different age groups, different sexes post exposure to different DMDS concentrations. Age1 indicates flies of age 3-5 days old, age2 indicates flies of 7-9 days old, and age 3 indicates flies of 11-14 days old.

Table 14. Significant differences of Least Squares Means of remaining life expectancy between different combinations of sex, Concentrations and age groups post exposure. Adjustment *P* value for Multiple Comparisons: Tukey-Kramer.

Sex	Tests	AG	vs	Sex	Tests	AG	Estimate	SE	DF	t Value	Adj P
female	5	1	vs	female	5	3	1.44	0.13	2020	3.97	0.0156
female	0.5	1	vs	female	5	1	1.35	0.1	2020	3.96	0.016
female	5	1	vs	female	5	2	1.49	0.15	2020	3.93	0.0183
male	5	2	vs	male	5	3	1.69	0.23	2020	3.87	0.0228
male	5	1	vs	male	5	2	1.41	0.13	2020	3.67	0.0451
female	5	3	vs	male	5	3	1.6	0.21	2020	3.57	0.0626
female	0.05	1	vs	female	0.05	2	1.84	0.17	2020	6.63	<.0001
female	0.05	1	vs	female	0.05	3	2.01	0.19	2020	7.51	<.0001
female	0.5	1	vs	female	0.5	2	2.1	0.24	2020	6.47	<.0001
female	0.5	1	vs	female	0.5	3	2.29	0.21	2020	9.05	<.0001
female	control	1	vs	female	control	2	1.69	0.09	2020	9.84	<.0001
female	control	1	vs	female	control	3	1.97	0.11	2020	12.17	<.0001
female	control	1	vs	male	control	2	1.97	0.12	2020	10.88	<.0001
male	0.05	1	vs	male	0.05	2	2.35	0.3	2020	6.62	<.0001
male	0.05	1	vs	male	0.05	3	2.13	0.24	2020	6.58	<.0001
male	0.5	1	vs	male	0.5	2	2.03	0.23	2020	6.13	<.0001
male	0.5	1	vs	male	0.5	3	2.34	0.28	2020	7.03	<.0001
male	5	1	vs	male	5	3	2.39	0.29	2020	7.11	<.0001
male	control	1	vs	male	control	2	2.01	0.13	2020	11.22	<.0001
male	control	1	vs	male	control	3	2.55	0.18	2020	13.4	<.0001

Remaining life expectancy post exposure

The type 3 tests of fixed effects on fly remaining life expectancy post exposure to different DMDS concentrations were demonstrated (Appendix A-5). Taken as a whole, sex significantly explained the difference in flies' life expectancy post exposure to DMDS ($F_{1, 2} = 39.43$, $df1 = 1$, $df2 = 2020$, $\alpha = 0.05$, $P < .0001$) (Appendix A-5). Age is also significant in explaining the difference in flies' remaining life span post exposure as well ($F_{1, 2} = 268.74$, $df1 = 2$, $df2 = 2020$, $\alpha = 0.05$, $P < .0001$) (Appendix A-5). Flies exposed to 0.5 μg/ml DMDS had longer life span compared to exposure to other concentrations (Figure 25), however the difference was not statistically significant. After exposure to DMDS at 0.05 μg/ml, females flies showed marginally significant longer life span compared to males ($df = 2020$, t value = 2.99, $\alpha = 0.05$, $P = 0.056$), besides, after exposure to DMDS at 0.5 μg/ml, females flies also showed marginally significant longer life span compared to males ($df = 2020$, t value = 2.91, $\alpha = 0.05$, $P = 0.070$). Female flies at age 2-5 days old showed significant longer life span after exposure to DMDS at 0.5μg/ml compared to exposure at 5 μg/ml ($df = 2020$, t value = 3.96, $\alpha = 0.05$, $P = 0.016$) (Table 14). At age 11-14 days old, female flies showed marginally significant longer life span than males after exposure to DMDS at 5 μg/ml, ($df = 2020$, t value = 3.57, $\alpha = 0.05$, $P = 0.062$) (Table 14).

Discussion

L. sericata use carrion resource for feeding, mating and oviposition. As described before, DMDS is one of the best studied VOCs associated with carrion resource and it was indicated to have dose effect on flies' olfactory response (Liu, dissertation).

In previous study, I investigated the behaviors of flies of different physiological status and sexes in response to DMDS in order to understand the role of VOCs in regulating flies response to carrion resource as well as the ecological roles of flies of different sexes. The current study categorized flies based on their age range and sexes, and investigated the defecation spots number, remaining life expectancy and survivorship of flies of different categories after exposure to different concentrations of DMDS to understand how the detection of DMDS may affect the life history related traits of flies.

In this study, different age and sexes groups of flies were used since age and sex have been shown to affect blow flies' behaviors in response to carrion associated resource [74]. DMDS of concentrations 5 μg/ml, 0.5 μg/ml and 0.05 μg/ml were used during the bioassay. These concentrations were chosen in reference to documented research [77] as well as based on my previously research on flies' olfactory response (Liu, dissertation chapter 3).

Flies' of different age groups and sexes showed different defecation post exposure to DMDS. At concentration of 0.5 μg/ml, flies of age 7-9 days old showed higher defecation compared to other tested DMDS. Also, my previous research showed that in response to DMDS at 0.5 μg/ml, flies of different biological status showed

significant behaviors, in which female flies were significantly more repelled to DMDS than males. While in the current study, females of age 7-9 days old post exposure to DMDS at 0.5 μg/ml had highest defecation, besides, flies exposed to DMDS at 0.5 μg/ml also had the longest life span compared to other concentrations (Figure 3). These results indicate the possible relation between blow flies' olfactory response and their life history related traits in terms of defecation, remaining life expectancy post exposure and survivorship. A described before, that DMDS indicate the availability of carrion resource, thus it may be concluded that the perception of this cue affects the defecation and aging of blow flies.

Life history traits, such as developmental time and body size [219], have been intensively studied to demonstrate effect of genetic and environmental factors [149]. However, insect defecation is relatively neglected in regard to its role as indicators of genetic and environmental effect. Although the odor cues, and the abilities to detect and response to these odors may differ in different species, the biological impact of the perceived information may be evolutionarily conserved [154].

The current study using DMDS as one of model VOCs, and defecation as a biological criterion to study studies' response to carrion associated VOCs, will help to understand insect behaviors in carrion ecology, and will help to develop new management for insect control and disease control, since pathogens can escape insect' immune system and excreted outside into the environment through defecation [214]. These pathogens can be transported in the environment and cause human disease [214].

Understanding how VOCs affect insect's defecation is important, since while using VOCs for pest control, it also important to consider its impact on defecation behaviors.

In a forensic context, understanding the age, sex and the concentrations of VOCs on the effect of blow flies' life history related traits will help to better estimate the physiological and biological status of the insect collected from the carcass, thus will lead to a more precise prediction about the time of the death of the remains. Such knowledge will become increasingly more important as we learn more about the role of insects in postmortem microbial composition and the effects of microbes on the biology of colonizing flies.

Insect feces was indicated to regulate insect behaviors through VOCs emitted by the microbial fauna released from gut [220]. Thus feces may also work as a conduit to regulate the microbial compositions in and outside of insect in response to their environment or as adapt to different biological status. The current study may provide stepping stone for further investigation into the microbial compositions of feces in response to perception of carrion associated VOCs, so as to complete our knowledge of interactions between blow flies with its biotic and abiotic environmental factors that are associated with carrion resource. Future study may carry out to use more physiological criterions such as fecundity, the change of compositions of symbiont microbiota inside insect post exposure to VOCs etc.

Further investigation into the role of olfaction in insect' physiology and behaviors may lead to the development of pest control based on insect olfaction, insect

nutritional and chemical ecology. Insect genetics may also contribute to the different defecations [215]. Taken as a whole, the observations from current study are likely the results of genetic variations associated with olfaction systems, metabolisms that lead to the varied defecation spots number and remaining life expectancy.

CHAPTER VI

SUMMARY AND CONCLUSION

The current experiments focused on, 1) developing tools to track *Providencia* through the fly; 2) the roles of volatile organic compounds emitted from carrion in regulating the olfactory response of blow flies, *Lucilia sericata* (Meigen) (Diptera: Calliphoridae); 3) the effect of olfaction of carrion associated volatile on the life history related characters of adult blow flies; and 4) the consequences of maternal choice and larval behavior on larval performance. Microbes play important roles in carrion decomposition. They act as nutrient recyclers to liberate nutrients such as essential amino acids to ecosystem for insect larval consumption by enzymatic digestion of the dead animal tissues [128], and they may also directly contribute to insect nutrition by production of essential amino acids [116-118]. The microbial degradation process during decomposition is accompanied by the releasing of a suit of VOCs, which may attract or repel insect.

Previous studies demonstrated some aspects of the interkingdom relationship between *L. sericata* and *P. mirabilis* which was originally co-isolated with *Providencia* from *L. sericata* salivary glands [67, 74]. It demonstrated that swarming deficient *P. mirabilis,* mutants that are deficient in QS signaling pathways, results in reduced *L. sericata* attraction and oviposition [74]. *Proteus* and *Providencia* were historically placed in a same tribe called *Proteeae* due to their similarity in phenotypical traits [163]. It is possible that interkingdom interactions may also exist between *L. sericata* and the

associated *Providencia* spp. I thus constructed a series of *gfp*-tagged *Providencia.* These *gfp*-tagged bacteria can be used as tools for further investigation into the interactions between *Providencia* species and *L. sericata*.

DMDS, indole and isobutylamine are by-products derived from the essential amino acids of methionine/cysteine [86, 92], tryptophan [104], and valine [111], respectively, and they are associated with carrion resources. *Proteus* and *Providencia* spp. are among the microbes that break down, and synthesize methionine [86, 92], and they are the two bacteria that associated with carrion and *L. sericata* [67]. PAA is an antibacterial agent produced by *Proteus* [53], can be derived from essential amino acid, phenylalanine, through bacterial degradation [115]. Thus, these VOCs mentioned above may indicate the presence of carrion resource as well as the associated microbes.

Although, DMDS, indole and isobutylamine are among the differentially regulated microbial emissions that potentially associated with variable blow fly responses [67, 74], there is still very limited about 1) how blow flies respond to these VOCs 2) how these VOCs may affect the physiology of blow flies during their exploitation of carrion resource 3) why flies choose the carrion resource and 4) what are the consequences of adult fly decision makings in carrion utilization, and how larvae perform on carrion resource as a result of both maternal decisions and their own strategies of carrion utilization.

During the investigation of response of *L. sericata* to VOCs associated with carrion/ larval resource, I assessed four VOCs: DMDS, PAA, indole and isobutylamine, to test how the different doses of volatile compounds affect fly response to these VOCs.

One of the most important findings was that flies of different sexes and physiological states exhibited different responses to different dose of the tested VOCs. For example, the observation that gravid females showed higher repellence to DMDS compared to other nongravid females and males, clarifies observations made by Frederickx [77] and helps to define attraction to a resource as a function of different ecological roles of different fly states. It may be possible that gravid females look for more quality resource for oviposition, and the DMDS concentration tested in the study did not dictate quality resource but carrion that has been decomposing for a few days, thus it is less attractive to gravid female *L. sericata*.

One previous study used DMDS to study its attractiveness on *L. sericata* [77]. This study provided a good stepping stone for behavior study in the related area, however the limitations in the study were that it did not categorize flies based on their sex and physiological status, and their chi-square statistical analysis does not allow to analyze more than one factor that might affect fly response to VOC. As demonstrated in my study, gravid females and non-gravid females showed significantly different response to different DMDS doses, thus not taking into account the types of flies will make the result open to biases. For instance, in the chi-square analyses non-gravid females in their study may have been the major group that led to their report that *L. sericata* is attracted to DMDS, causing them to miss the fact that gravid females appear to be uninterested in DMDS.

Blow flies exploit resource using VOCs released from decomposing tissues degraded by microbes, and the perception of these cues might in turn affect blow fly

physiology. DMDS has been previously identified as one of carrion associated VOCs [67, 74], however its effect of carrion associated the life history related traits of *L. sericata* was unstudied until now.

In my experiment, defecation, remaining life expectancy were used as life history related criterions to investigate the effect of exposure to DMDS on *L. sericata* adult biology. The results indicate the possible relationship between blow fly olfactory response, and their physiology through the perception of cues indicative of the availability of a food resource. Microbes associated with insects and their waste products may play an important role in ecological interactions with insects through defecation [221]; however insect defecation is relatively less studied compared to other life history related traits. Defecation might be associated with competition for resource for oviposition and food consumption, in which insects use cues from defecation to assess the environment [222]. Fecal material may also play a role in insect reproduction, as it may affect mate attraction etc. [223]. Furthermore, fecal pellets provide a source of mutualist microbes to larvae through ingestion, which allows transfer of gut/mutualist microbes from adult to larvae [224]. My study showed that flies of age 7-9 days old had the highest number of defecation spots compared to flies of age 3-5 days old, 11-14 days old and males once exposed to DMDS. Considering the different ecological roles of flies of different sexes and physiological status, it is possible that detection of VOCs from carrion may elicit and affect a series of physiological responses including defecation, through which process fly may adjust the microbiota environment inside their body so as to prepare for further activities such as food consumption, mating and oviposition once

reach the carrion resource. In addition, the results reported here indicate that there is a possibility that flies are seeding carrion with bacteria, possibly so that it may help inhibit the growth of potential competitors such as microbes and other microphagous animals that also feed on carrion resource. Thus, *L. sericata* may act as a mediator to respond and regulate the "trans-kingdom interactions" between bacteria inside and outside their body. My results indicate that defecation behavior is a potentially important trait to investigate environmental factors on insect physiology, providing a new dimension to study insect biology, ecology and evolution.

Carrion resources are ephemeral, lasting only a few days to a few weeks [149], and are also heterogeneous in terms of space, temperature and nutrition [159]. The strategies for choosing a resource is critical for larvae to survive, since they must identify and utilize the resources quickly before it disappears via consumption by other competitors (arthropods and microbes) [149]. Amino acids are important factors for insect nutrition [186]. Since the VOCs flies use to evaluate resources are derived from amino acids, it is possible that they use VOCs to inform them of the nutritional quality of remains that are potential resources for their offspring. In this study, when given the choices of four different diet that are deficient in one of the essential amino acids, *L. sericata* larvae showed most preference to diet without methionine, and least preference to diet without tryptophan. However when antimicrobials were added to the diets, larvae showed avoidance of the diet without methionine. It is possible that the addition of antimicrobials reduced methionine producing *Proteus* densities thus resulting in reduced production of QS molecules. The current investigation of larval performance on the diets

that mimic the carrion resource with different deficiencies of essential amino acids may provide a stepping stone for understanding the strategies of larvae in ephemeral and patchy resource utilization when faced with limited choices in their living environment.

There are several applications that can be derived from the current experiments. First, in the carrion ecology: the response of *L. sericata* to the carrion resource is associated with a suite of biotic and abiotic factors, including the patterns volatile organic compounds, which may be differed according to carrion types and their associated microbes. The study of blow flies response to different VOCs (Chapter 3) will provide a stepping stone for further studying response of the fly to more complicated and mixed VOCs associate with the carrion resource in the real world. Second, the presence of the bacteria might affect the pre-colonization interval [149], thus affect the estimation of the time of death of the remains [149]. Such information may be used to develop more precise estimates of colonization of remains (chapter 3). In addition, understanding the age, sex and the concentrations of VOCs on the effect of blow flies' life history related traits (chapter 5) will help to better estimate the physiological and biological status of the insect collected from the carcass, and may help to have a more precise prediction about the time of the death of the remains. For instance, the results presented here suggest that DMDS may play a role in the distribution of gravid and non-gravid females in attending remains during the summer in Texas [225]. Third, in insect pest management, understanding the olfactory (chapter 3) and physiological/ biological response (chapter 5) of blow flies to different carrion associated VOCs also will help develop a new management based on insect chemical ecology. It may be possible to interrupt the

interaction between the fly and the potential pathogens that they carry.

Understanding larval nutrition ecology and the role of symbionts in choosing and utilizing the resource (chapter 4) would suggest that it may be possible to bias the microbial compositions inside of insects, which may be utilized to kill insects. Fourth, in disease ecology, it is known that many pathogens can escape insect' immune system and be excreted outside into the environment through defecation, and can be transported in the environment and cause human disease [214]. Understanding how VOCs affect insect's defecation is important, since while using VOCs for pest control, it also important to consider its impact on defecation behavior since it may result in excretion of potential pathogens into the environment.

My work has provided a foundation for future work including: 1, Using *gfp*-tagged *Providencia* to study how interkingdom interactions impact the physiology and behaviors of *L. sericata* and its symbionts. 2, Using information such as behavioral and life history related physiological responses of *L. sericata* to carrion associated VOCs to study or predict the time of death of the remains. 3, To study if specific symbionts or microbes that are present on carrion impact larvae performance and survival. 4, The change of compositions of microbiota associated with defecation post exposure to VOCs. My research results indicate that the interactions between insect and microbes may be more complicated than we have realized. In addition to the interkingdom interactions between insect and microbes present in the carrion resource, *L. sericata* may act as a mediator in the "Trans-kingdom" interactions between the microbes in and outside their body. Further work may clarify the hidden roles of microbes in insect

chemical and nutritional ecology.

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APPENDIX A

Table A1. Type 3 tests of fixed effects

Tested VOCs	Effect	Number of degree of freedom	Denominator degree of freedom	F Value	Pr > F
DMDS	Dose	3	480	1.44	0.2299
	Type	2	480	0.89	0.4116
	Test	1	480	0.91	0.3407
	Type*Test	2	480	0.25	0.7813
	Dose*Test	3	480	0.69	0.5605
	Dose*Type	6	480	0.31	0.9341
	Dose*Type*Test	6	480	2.08	0.0537
Indole	Dose	2	324	0.09	0.9172
	Type	2	324	0.31	0.7323
	Test	1	324	1.28	0.2596
	Type*Test	2	324	0.14	0.8709
	Dose*Test	2	324	7.17	0.0009
	Dose*Type	4	324	0.09	0.9865
	Dose*Type*Test	4	324	0.94	0.4381
PAA	Dose	2	358	0.37	0.6903
	Type	2	358	0.12	0.8836
	Test	1	358	6.33	0.0123
	Type*Test	2	358	4.40	0.0130
	Dose*Test	2	358	0.14	0.8726
	Dose*Type	4	358	0.14	0.9685
	Dose*Type*Test	4	358	1.43	0.2232
Isobutylamine	Dose	2	342	0.14	0.8673
	Type	2	342	0.87	0.4184
	Test	1	342	1.08	0.2991
	Type*Test	2	342	7.23	0.0008
	Dose*Test	2	342	1.50	0.2252
	Dose*Type	4	342	0.77	0.5479
	Dose*Type*Test	4	342	2.45	0.0459

Table A-2. Type 3 tests of fixed effects on defecation post exposure to DMDS

Days post exposure to DMDS	Effect	Number of degree of freedom	Denominator of Degree of freedom	F Value	Pr > F
Day1	Sex	1	1269	282.90	<.0001
	Tests	3	1269	2.92	0.0331
	AG	2	1269	100.53	<.0001
	Sex*AG	2	1269	39.31	<.0001
	Tests*AG	6	1269	14.76	<.0001
	Sex*Tests	3	1269	12.90	<.0001
	Sex*Tests*AG	6	1269	19.76	<.0001
Day2	Sex	1	735	139.01	<.0001
	Tests	3	735	7.77	<.0001
	AG	2	735	41.19	<.0001
	Sex*AG	2	735	31.63	<.0001
	Tests*AG	6	735	3.27	0.0035
	Sex*Tests	3	735	6.46	0.0003
	Sex*Tests*AG	6	735	8.03	<.0001
Day 3	Sex	1	323	9.54	0.0022
	Tests	3	323	3.26	0.0219
	AG	2	323	8.00	0.0004
	Sex*AG	2	323	11.13	<.0001
	Tests*AG	6	323	3.80	0.0011
	Sex*Tests	3	323	4.81	0.0027
	Sex*Tests*AG	6	323	4.41	0.0003
Day 4	Sex	1	139	5.40	0.0215
	Tests	3	139	2.36	0.0737
	AG	2	139	14.06	<.0001
	Sex*AG	1	139	7.70	0.0063
	Tests*AG	2	139	1.77	0.1748
	Sex*Tests	3	139	1.42	0.2398
	Sex*Tests*AG	1	139	3.80	0.0533

Table A-2. Continued.

Days post exposure to DMDS	Effect	Number of degree of freedom	Denominator of Degree of freedom	F Value	Pr > F
Day 5	Sex	1	65	0.16	0.6919
	Tests	3	65	4.23	0.0086
	AG	1	65	2.08	0.1537
	LS	1	65	23.28	<.0001
	Sex*AG	0	.	.	.
	Tests*AG	0	.	.	.
	Sex*Tests	3	65	1.27	0.2924
	Sex*Tests*AG	0	.	.	.
Day 6	Sex	1	25	4.44	0.0452
	Tests	2	25	1.80	0.1855
	AG	0	.	.	.
	Sex*AG	0	.	.	.
	Tests*AG	0	.	.	.
	Sex*Tests	3	25	3.98	0.0191
	Sex*Tests*AG	0	.	.	.
Day 7	Sex	0	.	.	.
	Tests	0	.	.	.
	AG	0	.	.	.
	Sex*AG	0	.	.	.
	Tests*AG	0	.	.	.
	Sex*Tests	1	8	0.05	0.8334
	Sex*Tests*AG	0	.	.	.
In total	Sex	1	2019	167.37	<.0001
	Tests	3	2019	1.16	0.3235
	Sex*AG	2	2019	56.4	<.0001
	Tests*AG	1	2019	26.32	<.0001
	Sex*Tests	2	2019	11.44	<.0001
	Sex*Tests*AG	6	2019	2.35	0.0280

Table A3. Estimated number of defecation spots ± SD of *L. sericata* adults at different days post exposure to DMDS at 0.05 µg/ml, 0.5 µg/ml and 5 µg/ml. Exposure to DMDS was performed at 20ºC -23ºC and on a 12:12 L: D photoperiod for 24 hours.

Day post exposure	DMDS Concentration (µg/ml)	AG	sex	estimated number of defecation spots ± SE
Day 1	0.05	1	Female	10.89 ± 0.73
			Male	11.43 ± 0.94
		2	Female	9.18 ± 0.51
			Male	7.28 ± 0.50
		3	Female	8.69 ± 0.35
			Male	4.80 ± 0.47
Day 1	0.5	1	Female	13.28 ± 0.72
			Male	9.75 ± 0.58
		2	Female	19.30 ± 0.89
			Male	7.79 ± 0.51
		3	Female	10.16 ± 0.48
			Male	6.85 ± 0.44
Day 1	5	1	Female	10.89 ± 0.61
			Male	10.33 ± 0.66
		2	Female	5.51 ± 0.40
			Male	8.34 ± 0.51
		3	Female	12.85 ± 0.52
			Male	5.79 ± 0.39

Table A-3. Continued.

Day post exposure	DMDS Concentration (µg/ml)	AG	sex	estimated number of defecation spots ± SE
Day 2	0.05	1	Male	8.47 ± 0.67
		2	Female	12.55 ± 0.74
			Male	3.99 ± 0.60
		3	Female	7.67 ± 0.58
			Male	3.64 ± 0.51
Day 2	0.5	1	Female	17.23 ± 0.91
			Male	10.27 ± 0.85
		2	Female	8.72 ± 0.71
			Male	6.12 ± 0.65
		3	Female	6.99 ± 0.53
			Male	3.44 ± 0.54
Day 2	5	1	Female	9.49 ± 0.66
			Male	11.16 ± 0.66
		2	Female	7.45 ± 0.55
			Male	7.77 ± 0.66
		3	Female	8.87 ± 0.55
			Male	3.66 ± 0.53
Day 3	0.05	1	Female	7.48 ± 0.50
			Male	7.94 ± 0.61

Table A-3. Continued

Day post exposure	DMDS Concentration (µg/ml)	AG	sex	estimated number of defecation spots ± SE
Day3	0.05	2	Female	11.12 ± 1.74
			Male	1.98 ± 1.16
		3	Female	10.48 ± 1.74
			Male	3.36 ± 1.01
Day 3	0.5	1	Female	6.98 ± 0.84
			Male	6.59 ± 0.72
		2	Female	5.35 ± 1.03
			Male	3.31 ± 0.85
		3	Female	11.70 ± 1.96
			Male	4.99 ± 1.49
Day 3	5	1	Female	6.63 ± 0.76
			Male	8.37 ± 0.60
		2	Female	7.43 ± 0.95
			Male	9.18 ± 1.03
		3	Female	11.52 ± 1.29
			Male	2.35 ± 1.68
Day 4	0.05	1	Female	5.23 ± 0.57
			Male	5.43 ± 0.70
Day 4	0.5	1	Female	5.82 ± 0.67

Table A-3. Continued.

Day post exposure	DMDS Concentration (µg/ml)	AG	sex	estimated number of defecation spots ± SE
Day 4	0.5	1	Males	5.93 ± 0.82
Day 4	5	1	Female	6.08 ± 0.94
			Male	6.04 ± 3.50
		2	Female	5.78 ± 0.84
			Male	4.14 ± 1.47
Day 5	0.05	1	Female	5.04 ± 0.81
			Male	4.82 ± 1.10
Day 5	0.5	1	Female	5.24 ± 0.84
			Male	2.62 ± 0.66
Day 5	5	1	Female	10.32 ± 1.62
			Male	8.16 ± 1.60
Day 6	0.5	1	Female	7.77 ± 1.67
			Male	5.37 ± 2.75
Day 6	5	1	Female	4.85 ± 1.58
			Male	2.49 ± 1.69
Day 7	0.5	1	Female	6.40 ± 1.62

Table A-4. Differences of Least Squares Means. Number of defecation spots were compared over the length of the experiment without day effect.

Sex	Tests	AG	vs	Sex	Tests	AG	Estimate	Standard Error	DF	t Value	Adj P
female	0.05	1	vs	female	0.05	2	-0.1502	0.1226	2019	-1.23	0.9999
female	0.05	2	vs	female	5	2	0.5071	0.1244	2019	4.08	0.0103
female	0.05	2	vs	male	0.05	2	0.5594	0.1399	2019	4	0.014
female	0.05	3	vs	male	0.05	3	0.5644	0.1326	2019	4.25	0.005
female	0.5	1	vs	male	0.5	1	0.389	0.1114	2019	3.49	0.0808
female	0.5	2	vs	female	5	2	0.6943	0.136	2019	5.11	<.0001
female	0.5	2	vs	male	0.5	2	0.6424	0.1415	2019	4.54	0.0015
female	0.5	3	vs	male	0.5	3	0.4968	0.1315	2019	3.78	0.0316
female	5	2	vs	female	control	2	-0.4266	0.1105	2019	-3.86	0.0234
female	5	3	vs	male	5	3	0.6223	0.1281	2019	4.86	0.0003
female	control	2	vs	male	control	2	0.3719	0.09619	2019	3.87	0.023
female	control	3	vs	male	control	3	0.5803	0.1039	2019	5.58	<.0001
male	0.05	1	vs	male	0.05	2	0.3278	0.1571	2019	2.09	0.9191
male	0.05	1	vs	male	0.05	3	0.5693	0.1467	2019	3.88	0.0217
male	0.05	1	vs	male	control	1	-0.01869	0.09845	2019	-0.19	1
male	0.05	2	vs	male	0.05	3	0.2415	0.1542	2019	1.57	0.9974
male	0.05	2	vs	male	0.5	2	-0.1041	0.1576	2019	-0.66	1
male	0.05	2	vs	male	5	2	-0.1657	0.1526	2019	-1.09	1
male	0.05	2	vs	male	control	2	-0.107	0.1413	2019	-0.76	1

Table A-4. Continued.

Sex	Tests	AG	vs	Sex	Tests	AG	Estimate	Standard Error	DF	t Value	Adj P
male	0.05	3	vs	male	0.5	3	-0.1346	0.1539	2019	-0.87	1
male	control	1	vs	male	control	3	0.4578	0.1022	2019	4.48	0.0019
female			vs	male			0.3623	0.0344	2019	10.53	<.0001

Table A-5. Type 3 tests of fixed effects of life remaining life expectancy.

Effects	Number of degree of freedom	Denominator degree of freedom	F value	Pr > F
Sex	1	2020	39.43	<.0001
Test	3	2020	0.85	0.4643
AG	2	2020	268.74	<.0001
Sex*AG	2	2020	4.64	0.0098
Test*AG	6	2020	3.23	0.0037
Sex*Test	3	2020	0.79	0.4986
Sex*Test*AG	6	2020	2.01	0.0612