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Bacterial Volatiles as Potential New Attractants for the Island Fly (*Dirioxa pornia*) (Tephritidae: Phytalmiinae)

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DECLARATION OF ORIGINALITY

I, Nilesh A. Chand, declare that this work contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

N A Chand

Nilesh A. Chand

Date: 9th October 2017

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TABLE OF ABBREVIATIONS

ADI	Acceptable daily intake
APVMA	Australian Pesticide and Veterinary Medicines Authority
CSIRO	Commonwealth Scientific Industrial Research Organisation
EAD	Electroantennogram detector
EAG	Electroantennography
GC-EAD	Gas chromatography-electroantennogram detection
GC-FID	Gas chromatography-flame ionisation detector
GC-MS	Gas chromatography-mass spectrometry
MAT	Male annihilation technique
Medfly	Mediterranean fruit fly
MT	Mass trapping
MVOC	Microbial volatile organic compounds
NSW	New South Wales
Q DPI	Queensland Department of Primary Industries
QFly	Queensland fruit fly
PBS	Phosphate buffered saline
SARDI	South Australian Research & Development Institute
SIT	Sterile Insect Technique
TD	Thermal desorption

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ABSTRACT

Many fruit fly species have a detrimental impact on food production. Lure and kill and mass trapping methods are becoming more important for crop protection, but currently, Island Fly (*Dirioxa pornia*) does not have a specific attractive lure for males and females. This study is initial research into olfactory responses to gut bacteria emissions.

Fruit flies use olfactory cues for locating bacteria that assist in their development cycle. The ingestion of food together with naturally occurring bacteria allows a microbiome to form in their intestinal tract. This project investigated the gut microbiome of laboratory reared Island Flies, the chemical composition of the volatiles released from these microbes by GC-MS and the attractiveness of Island Flies to the volatile isolates using Y-tube olfactometry, electroantennography and wind tunnel experiments.

All bioassay results of Island flies showed a rapid attraction towards the identified bacterial volatile isolates of *Bacillus sp*. and deterrence towards *Citrobacter freundii*. Other bacterial isolates of *Bacillus subtilis, Klebsiella oxytoca* and *Providencia rettgeri* showed low to moderate attractions respectively. *Bacillus sp.* uniquely had alkanes, some alcohols, aldehydes and nitrogen containing compounds while *C. freundii* had many sulphur-containing compounds and acids. Further studies on these compounds may assist in the development of a microbial-based lure.

CHAPTER 1. INTRODUCTION

1.1 Fruit Flies – Life Cycle and Economic Importance

The Food and Agricultural Organisation of United Nations reports that several species of fruit flies, like Mediterranean Fruit Fly (*Ceratitis capitata*), Mexican Fruit Fly (*Anastrepha ludens*), West Indian Fruit Fly (*A. obliqua*), Oriental fruit fly (*Bactrocera dorsalis*) and the South American Fruit Fly (*A. fraterculus*) have a much greater impact on global agriculture and trade than any other known pests. Fruit flies are a globally devastating biosecurity risk, being insect pests of horticultural crops, and causing billions of dollars in crop production losses annually [1]. They have an impact upon horticultural production and market access. Countries such as New Zealand, Japan, Chile and USA, which are currently free of key damaging fruit fly pests, would face serious social and economic consequences if these pests were introduced [2]. With the global changes in climate (global warming), introduced pests including fruit flies would have a greater chance of surviving and spreading in otherwise previously inhospitable areas.

Australia's average worth for local and international trade of horticultural markets is $4.8 billion [3] and the production losses could add up to $159 million a year. The presence of economically important fruit flies in Australian horticultural zones also restricts international trade opportunities that are worth $500 million annually and places restrictions on interstate movement of fresh horticultural goods. Fruit flies can easily be transmitted from an infested to uninfested area with fresh fruits and vegetables in egg or larvae form and are very difficult to detect in the initial stages of their development.

The careful examination of infested produce will reveal visible sting marks where eggs have been deposited underneath the fruit peel and larval development will follow soon afterwards. The larvae develop in three instar stages within the fruit and vegetable tissues, feeding vigorously on the decaying fruit tissues. They turn into pupae and become the resting stage of the fruit fly life cycle. The pupae are enclosed within a puparium formed by the outer skin of the larvae that turns hard and tanned. When the moisture and temperature conditions are appropriate, the pupae hatch into adult flies by splitting open the puparium at the anterior end and squeezing out of it. The Island Fly *(Dirioxa pornia)* (Walker, 1849) (Tephritidae: Phytalmiinae) life cycle typically takes on average about 22-29 days to

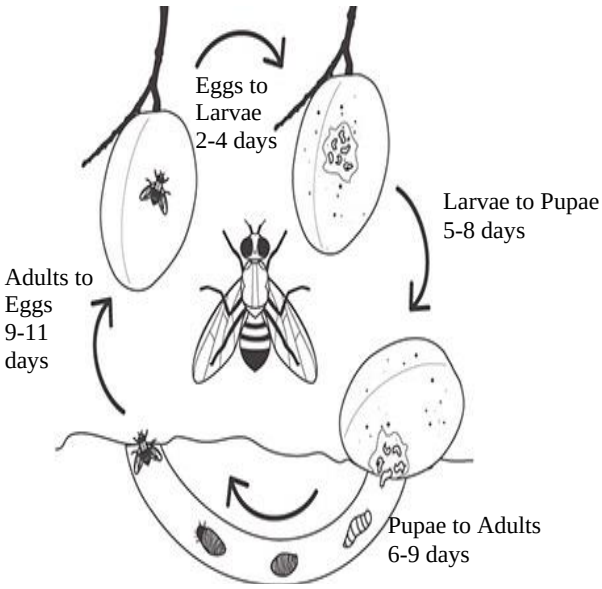

Figure 1: Typical Life Cycle of Island Fly

Source: Prevent Fruit Fly – Plant Health Australia [3]

complete under favourable conditions (Figure 1). The length of this cycle varies between different fruit fly species[4]. Thus, areas and countries free of fruit fly impose severe restrictions on fresh produce imports originating from or transiting through fruit fly prone areas *via* pre-and post-harvest treatments to render the produce free from fruit fly prior to shipments.

1.2 Control and Management

The control and management of fruit flies can be effectively carried out by having a good knowledge of its life cycle and exploiting its weakness in the development cycle. Australia has two economic species of fruit flies, the Mediterranean fruit fly (Medfly) (*Ceratitis capitata*) and Queensland fruit fly (Qfly) (*Bactrocera tryoni*). Qfly is an endemic Australian species and is found in New South Wales, Victoria, Northern Territory and Queensland. Medfly is an introduced species to Australia and occurs mainly in parts of Western Australia[3]. Both fruit fly species cause severe crop losses to host fruits and vegetables if not managed properly and a proper control and management procedure is necessary for horticultural industries to remain viable.

The main methods of control and management for fruit flies include orchard hygiene, protein baiting, mass trapping, cover spraying and scouting and male annihilation technique (MAT). Until recently, in Australia the most popular method was the cover spraying of pesticides directly on to host plants. The Department of Agriculture and Water Resources (DAWR) Australia has recently restricted the use of organophosphate systemic insecticides (fenthion and dimethoate - trade names Rogor and Lebaycid respectively)[5] in all commercial horticultural productions. These chemicals, being systemic and having a residual effect were effective in fruit fly control and management strategies adversely affecting any larval growth of fruit flies within the fruit tissues. These chemicals were restricted due to their indiscriminate effects of killing beneficial insects and upsetting Integrated Pest Management (IPM) programs, environmental effects of toxicity to wildlife, birds and fish, phytotoxic effects on some fruits and vegetables and adverse human health effects *via* repeated applications. While cover spraying of pesticides was effectively used to manage Qfly and Medfly in commercial horticulture, other pests of fruits and vegetables were also managed at the same time including Island Fly. In the absence of suitable chemical interventions, other methods must be adopted to control fruit fly populations and prevent their spread.

1.3 Alternative Control Options

Many alternative control options are available for the control and management of fruit flies. One of the alternative controls to chemical sprays is the lure and kill method, commonly known as Male Annihilation Technique (MAT). This method relies on the ability of a lure to attract male fruit flies to a substrate and for an insecticide present on that substrate to kill the flies upon contact. The

commonly used substrates, such as dental cotton wicks, particle board blocks, cardboard blocks and coconut husks are capable of holding the lure after being dipped in lure and impregnated with insecticide solutions for a period of time. Since the pesticide employed in the lure traps are enclosed in a container there is very little risk of the chemical insecticide contaminating the environment or affecting humans and other animals. There are also naturally derived chemicals like Spinosad®, which are available and are derived from soil microbes that can be alternatively used with exposed substrates. Nonetheless, many fruit fly species still do not have specific lures developed to control and manage them.

Sterile Insect Technique (SIT) involves the production of male fruit flies that are sterilised with low radiation and released periodically in an area to copulate with wild females but are unable to produce viable offspring. SIT could be an option as an alternative control of fruit flies and is often combined with Area Wide – Integrated Pest Management (AW-IPM) that consists of lure and kill methods as well to initially reduce the fruit fly population from both horticultural areas and refugia. The chances of insects becoming resistant is negligible because the SIT is a species-specific approach.

1.4 Island Fly

Figure 2: Adult Island fly - Female

(C) Atlas of Living Australia [6]

Island Fly (*Dirioxa pornia*) (Walker, 1849) (Tephritidae: Phytalmiinae) (Figure 2) is one such species that does not have a known specific lure. It was previously referred to scientifically as *Trypeta pornia* (Walker, 1849)*; Rioxa (Dirioxa) confusa* (Hardy, 1951) *and Trypeta musae* (Froggatt, 1899)[6]. It is classified in the Animalia kingdom, Arthropoda phylum, Insecta class, Diptera order and Tephritidae family. Island Fly is a cosmopolitan native Australian species and also found in New Caledonia [7].

It is distributed in all Australian states except for Tasmania (Figure 3). It extends in Eastern Australia, from Iron Range, Cape York Peninsula, to southern New South Wales and was also introduced to Perth, Western Australia as well as Northern Victoria[6]. Island Fly has always been and is considered as a non-economic species of fruit fly in Australia as it does not readily attack sound fruits and vegetables [8]. This is probably due to its shorter and blunt ovipositor in comparison to other economic fruit fly species. It lays its eggs in over-ripe, damaged and fallen or partially decomposing fruits and vegetables including citrus. Island Fly has been intercepted by overseas quarantine agencies in the last decade on shipments of citrus (Navel

and Valencia varieties) from Australia especially to Japan [9], USA and New Zealand [8]. The interception of *D. pornia* eggs and larvae in November 2000 in New Zealand resulted in a one week suspension of citrus trade, until the larvae hatched and were identified [10]. Qfly and Island Fly have very similar larvae and can be safely distinguished by rearing into adult flies. Australia's major trading partner countries such as USA, New Zealand and China have considered it as an actionable quarantine pest in their import pathways assessments. Island Fly's hosts include a wide range of plant families such as: *Anacardiaceae, Annonaceae, Araucariaceae, Capparaceae, Caricaceae, Clusiaceae, Combretaceae, Curcurbitaceae, Ebenaceae, Euphorbiaceae, Fabaceae, Lauraceae, Lecythidaceae, Loganiaceae, Moraceae, Musaceae, Myrtaceae, Oleaceae, Oxalidaceae, Passifloraceae, Proteaceae, Rosaceae, Rubiaceae, Rutaceae, Sapindaceae, Sapotaceae, Solanaceae* and *Xanthophyllaceae* [7]. Thus, with this wide range of hosts that almost covers the entire Australian export produce market, a proactive research into the management and control of this pest is needed. Research activities will provide confidence to trading partners on an ongoing basis of the preventative measures that are being developed to address the issues associated with these hosts of *D. pornia*.

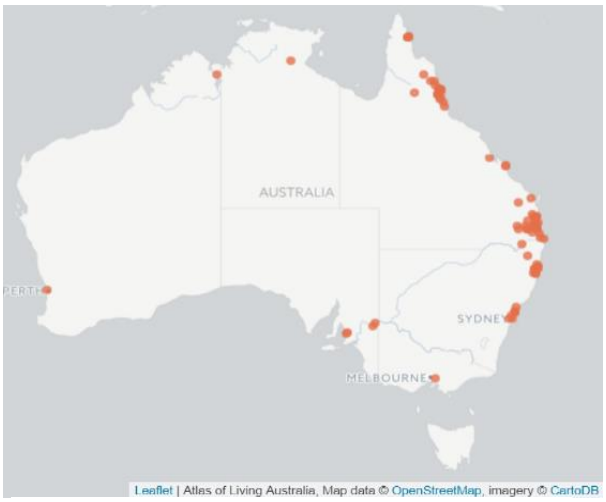

Figure 3: Island Fly occurrences in Australia

(C) Atlas of Living Australia [2]

Since the ban of the organophosphates in Australian horticultural areas, there are no control measures currently available to manage Island Fly populations. With the application of alternative control measures for Medfly and Qfly *via* MAT and SIT, the population of Island Fly is expected to grow due to reduced competition for space and resources from these two fruit fly species. Island Fly shares similar food sources such as over-ripe or fermented produce as *Drosophila suzuki* [11] and was very recently reclassified as a devastating economic pest. With the lack of field control and management for Island Fly and availability of more resources, its population is expected to rise rapidly and cause adverse effects as well.

As Island Fly is not currently considered an economic species in Australia its damage losses are not quantified, but the interception of Island Fly in export consignments on fresh produce from Australia represents a huge loss in the international market. This loss is likely to have a trickle-down effect to all the players involved in the pathway from the producers to the importers. Furthermore, the imposition of trade bans could be a costly exercise to negotiate and re-open the trade pathways with involvement of substantial time that the Australian horticultural industries cannot afford. For

example, in 1995 Asian papaya fruit fly, *B. papayae* was detected near Cairns, Queensland. The impact on Australian industries was estimated to be $100 million for lost trade opportunities and additional treatment costs with expenditures of $36M to eradicate it [3].

In addition to the current threat posed by fruit flies in prone areas, the geographical range of fruit flies generally in Australia will likely broaden due to the increase in global temperatures [12]. Thus, the historically cooler regions of Australia being fruit fly free zones could become conducive and prone to fruit flies. This climatic change combined with the apparent lack of control for fruit flies in orchards could lead to high population numbers causing outbreaks and subsequent increase in losses.

1.5 Island Fly – Background Work

This project aimed to rear and establish an Island Fly colony in the laboratory. There is only been one other successful attempt by Dr Peter Crisp's laboratory in SARDI, South Australia [13]. Other researchers (Morrow [14], Riegler [14], Schutze [15], Jessup [16], Royer [17] and Lee [18]) attempted to raise Island Fly in their respective laboratories but achieved little success and thus abandoned any further work on Island Fly. Thus, there are very few publications concerned with Island Fly (*D. pornia*). The few publications on Island Fly consists of Morrow *et. al.* [11] project using a single Island Fly from the wild in their assays. They investigated on Island Fly's gut microbiome together with other *Bactrocera* species and *C. capitata*. Other publications mention the occasional captures of *D. pornia* in traps [19-21] were noted during distribution surveys for other fruit fly species.

1.6 Gut Microbiome

Island Flies feed primarily on available nutrients in their habitats, from sources of fruits, honey dew and bird droppings. While scavenging for feed, they encounter many types of bacteria in their surroundings. Some species of bacteria are beneficial to them while others are not. They are able to differentiate the areas having beneficial bacterial species through their olfactory senses on their antennae [88]. While feeding, the flies are also able to ingest the beneficial bacteria species that assists in their lifecycle. A colony is formed in their alimentary canal together with the bacteria inherited from their parents that will be present throughout their lifecycle. It has been observed in the SARDI laboratories that Island Flies fed with a certain species of bacteria (*Bacillus sp.*) perform better in the laboratory cultures than those without it [13]. The bacteria fed flies had greater longevity, higher fecundity and lower death rates in cages. Thus, a symbiotic relation is formed between the flies and their beneficial bacteria; whereby the bacteria obtains a secure living space in the flies and gets transported to new environments and in turn benefits the flies' survival chances.

Many species of fruit flies have been studied for symbiotic association with their gut bacteria and include *Anastrepha, Bactrocera, Ceratitis* and *Rhagoletis* [11, 23-39]. Petri (1909) reported on gut

bacterial symbiosis for the first time for *Bactrocera oleae* (Rossi) (Olive Fly), and since then many other researchers followed [40]. Many different bacterial genera have been identified in fruit flies such as *Acetobacter, Agrobacterium, Arthrobacter, Bacillus, Citrobacter, Defluvibacter, Delftia, Enterobacter, Escherichia, Erwinia, Flavobacterium, Hafnia, Kluyvera, Klebsiella, Listeria, Lactobacillus, Micrococcus, Ochrobactrum, Pantoea, Pectobacterium, Pseudomonas, Proteus, Providencia, Staphylococcus, Stentrophomonas, Streptococcus, Raoultella, Serratia* and *Xanthomonas* [20, 23, 37, 38, 41-50]. These bacteria have a symbiotic relationship with their hosts. They are important for the host's developments, nutrition, resistance to pathogens, reproduction and semiochemicals production [29, 50, 51, 96]. The fecundity, survivability and consistent development are largely dependent upon the bacteria providing digestive enzymes or vitamins [30] that are not found in fruit tissues, ultimately improving digestion efficiency. Additionally, bacteria were found to degrade toxic compounds ingested by host insects and assist in building up insecticide resistance [52].

Furthermore, the functions of bacteria in insects' guts such as desert locusts and termites have also been studied considerably for mutualistic interactions [92, 93]. Parasitic interactions have been noted for bacterium, *Paenibacillus larvae* (American foulbrood) in honeybees [94].

Moreover, Morrow *et. al.* (2015) state that in their initial project for comparing microbiomes of field-caught and laboratory adapted Tephritid fruit flies of *Bactrocera* genus and *D. pornia* in Australia[11], that there was a turnover of microbial diversity within and between fruit fly species. They were able to detect streamlining of microbiome in laboratory reared flies in comparison to field collected ones. *Enterobacteriaceae* was found in all samples and in lower abundance in Island Fly; however, no bacteria genus was common to all Tephritid genera in their research. Island Fly had 93% of *Acetobacteraceae* bacterial community that is similar to *Drosophila* species also having fermentation driven ecological niches; while *Bactrocera* samples had relatively low abundance from 0 to 8.13% only. Also, the polyphagous species had more microbiota diversity than the monophagous species and the environment appeared to be the primary factor that shaped the bacterial community composition in fruit flies.

Schulz *et. al.* (2007) reported that deformations can be induced by *Bacillus subtilis* volatiles on phytopathogenic or clinically relevant fungi [53]. A symbiotic relationship with *B. subtilis* benefits the fruit fly in tackling fungus that enters the fruit fly primarily through feeding. Furthermore, they state that *C. freundii, K. pneumonia* and *Enterobacter agglomerans* produced volatiles that attracted the Mexican fruit Fly (*Anastrepha ludens*). These *Enterobacter* species are commonly found in faeces of birds and those faeces that contains these bacteria in them are more attractive to *A. ludens* than those that don't. These bacteria could be symbiotically linked to Mexican fruit fly for conversion of

unusable nitrogen compounds to usable ones or for production of odour emitting compounds in proteinaceous nutriments attractive to these flies [53].

As reported by Hadapad *et. al*. (2015) from a research on melon fly *B. cucurbitae* in India, flies from six different states had differing bacterial isolate species within them [54]. The core bacterial complement was almost identical, yet there were some new species from different states that could be attributed to variations in their food availability and environmental conditions. A similar result was obtained by Morrow *et. al*. with differing bacterial cultures from within and between fruit fly communities [11].

1.7 Olfaction Behaviour

Olfaction plays a major role in human food preferences, especially for products such as beer, wine, cheese and yoghurt. Similar is the case with microorganisms such as fungi and bacteria in nature with the production of microbial volatile organic compounds (MVOC). Davis *et. al.* (2013) state that complex chemosensory systems have been evolved by insects that are extremely sensitive to volatile chemical signals [55]. The insects' ability to use microbial emissions as semiochemicals for behavioural cues suggests their symbiotic associations.

Survival of insects in this universe has been linked to their remarkable capability of chemoreception and olfaction. For example, the silkworm moths could respond to 3000 molecules/ml of air [56]. There is good understanding of the cellular and molecular mechanisms of the outstanding sensitivity of insects' olfaction and is quite advanced as evident by the surge of recent publications. Common examples are the potential male tephritid attractant of Cuelure for Qfly and methyl eugenol for Oriental fruit fly being currently used for control and management of tephritids.

One of the control methods to accommodate reduced insecticide use is to exploit the olfactory responses of fruit flies to attractive odours. Although this seems a promising option, there is very little information and research in this regard especially for Island Fly. Cultivable gut bacteria are considered more beneficial than non-cultivable species of gut bacteria to prepare conducive fruit fly lures. Many studies on arthropods found that the insect olfactory system is highly selective and sensitive to semiochemicals (a chemical that conveys a signal from one organism to another to modify the behaviour of the recipient organism) and can discriminate specific odours to mediate important behaviours, including locating mates, food sources and oviposition (egg laying) sites [88]. This function is achieved by the sensillae on the antennae, which detect the semiochemicals to activate olfactory sensory neurons and translate the chemical signals into nerve impulses to the brain. A combination of native preferences and cognitive abilities gives insects and especially fruit flies, the

ability to engage in specialized interactions with their host environments while being flexible to adapt to their natural variability or to exploit new resources [90].

Fruit fly oviposition and feeding behaviour are influenced by various components of bacterial odours in their environment and these volatile properties have been used extensively in traps and baits for their management and control. Initial attractants used for luring fruit flies *via* their olfaction senses are protein food baits in traps [58, 59]. These proteins are able to attract fruit flies, as the protein is needed by them as a nutrition material especially for reproduction. However, the protein attractant formulations are unstable in the environmental conditions they were being used in and needed constant formulation and replenishments in the field. Consequently, more durable and practicable lures are needed [60]. Thus, with the knowledge that fruit flies are attracted to certain bacteria in the environment, this project was formulated on Island Fly, as a model fruit fly species, to investigate further on these aspects.

1.8 Volatile Organic Compound

Various bacteria produce volatile compounds that are the cues picked up by the fruit fly antennae. These semiochemicals or microbial volatile organic compound [55, 61] cues activate a signal to be sent from the sensillae on antennae to the fly's brain indicating the presence of volatiles. If their brain processes the volatile as a positive or favourable substance, the brain sends signals to the fly muscles to be attracted towards the bacterial site for further exploitation. However, if the cues are processed as negative, then the fly navigates away from the bacteria odours with similar opposing signal mechanisms. The odour is primarily responsible for the fly to act in a certain way towards different species of bacteria. The bacterial odour has certain components that are closely involved with fruit fly behaviour in stimulating oviposition or allowing for feeding that can be used in the management and control for trapping and baiting activities [91].

Other researchers state that the identification of new active volatile compounds in the semiochemical system of the olive fly [62] was promising for the development of innovative control strategies in area-wide pest management programmes. Thus, the innate olfactory responses of fruit flies to volatiles and especially to bacteria for mating and oviposition can be exploited by researchers for their control and management efforts.

1.9 Aims

The aims of this project and its scope were to:

i. identify Island Fly gut bacteria.

ii. characterise the volatile organic compounds emitted by Island Fly gut bacterial isolates.

iii. determine the effects of bacterial isolates on Island Flies (attraction or deterrent).

iv. compare male and female behaviour responses when exposed to the gut microbiome.

1.10 Hypothesis

The hypothesis of this project was that at least some bacterial isolates shall produce a semiochemical profile that Island Fly would find attractive, so that the bacteria could attract flies to them and be ingested, helping both the flies and the bacteria.

CHAPTER 2: EXPERIMENTAL METHODS

2.1 Island Fly Colony Rearing

Island Flies were reared at the Biological Sciences laboratory (W 19 N) of Macquarie University, Sydney, Australia from pupae in naturally infested citrus fruits collected in February 2017 from the Central Coast of NSW, Adelaide, South Australia, Canberra, ACT and from Brisbane, Queensland [16, 18, 63, 64]. Since this initial parental batch (F0) of Island flies were all obtained as pupae from the wild fruits and were being domesticated for laboratory rearing, it was necessary to provide them with conditions resembling their normal environment to prevent major disruptions to their life cycle. The flies were placed in 12.5 litre storage containers (Décor brand) that had a ventilation of 10 cm diameter openings on three of the six sides of the container. The three ventilation holes on the storage container were covered with fibreglass insect mesh wire (1.5 mm thickness). They were incubated in a controlled environmental room of 27^{o}C (± 1^{o}C); relative humidity of 70% (±10%) and photoperiod of 13:11 L:D. The lights in the room were adjusted to have a dawn and dusk period of 30 minutes at the start and end of the light phase. All adults fruit flies emerging from the incubated pupae were identified morphologically using The Australian Handbook for the Identification of Fruit Flies, Version 2.1 [65].

A larval gel diet (Appendix 1) modified from Chang's gel diet [66] for Oriental Fruit Fly and Tahereh's gel diet for Qfly [67] was prepared containing yeast and sugar. All the dry ingredients were carefully weighed and mixed together thoroughly in a mixing bowl. Organic wheat germ oil (Melrose Health Group, Australia), a rich source of fatty acids and vitamin E, was then added to the mixture. Addition of wheat germ oil to fruit fly diet improves hatching of eggs, percentage of adult flies, egg production and pupal recovery in some fruit flies [67, 68]. Wheat germ oil was not used previously in Island Fly diets [13] and has been included here in an attempt to improve the health of Island Fly laboratory colonies.

Boiling water was added to the mixture and it was blended thoroughly in a food mixer for 10-12 minutes, until a consistent paste was obtained. The boiling water activated the agar so that it would set into a gel media as the paste cooled [69]. 40 mL of the warm diet paste was poured into a 50 mL plastic dressing containers ('diet container'), which was then covered with a lid and allowed to cool to room temperature. Once the paste had cooled and gelled, a sterile pin was used to make 12 to 16 holes on the side of each diet container. The holes were made around the mid to the bottom half of the diet containers and the diameter of the holes was large enough to allow the Island Fly ovipositor to pass through.

The diet containers were placed with Island Flies in the clear 12.5 litre plastic cages containing 20-25 male and female Island flies. White sugar and yeast hydrolysate (3:1 ratio, MP Biomedicals, USA) was provided as adult food in Petri dishes and water was provided *ad libitum via* a wick attached to a 50 ml vial containing water [70].

Female Island flies were observed laying eggs directly into the holes in the diet containers. After a 24-hour exposure of the gel diet to the flies in the cages, the diet containers were removed and placed in a fruit fly cage (Décor brand, 12.5 litre with 3 meshed ventilators) containing a 2 cm layer of sterilised moist vermiculite. The fruit fly cages containing the diet containers were completely covered with another larger black container (41 litre storage container, Bunnings Warehouse, Australia). This ensured that the eggs had darkness to simulate a natural environment of a host fruit pulp. The moisture from the vermiculite simulated soil media in nature.

The containers were checked for egg hatching and additional moisture was added to the diet when needed *via* a spray bottle containing tap water. When the larvae reached the third instar stage and were "jumping out" of the diet gel, the lids of the diet containers were carefully removed. The larvae developed into pupae in the moist vermiculite and later eclosed as F1 adult Island flies (Figure 4) for use in assays. The F1 Island flies were kept in a separate rearing cage in the same rearing room as the parental cages. The F1 generation flies were also provided with food and water in a similar manner as to their parents. All food and water provided to the reared flies were renewed weekly. The F1 flies were transferred to clean fruit fly rearing cages every three weeks to prevent microbial build up in the cages.

Figure 4: Adult Island fly - Female

2.2 Island Fly Bacteria Extractions

When the Island flies were 10-14 days old, females of the F1 generation were dissected and bacteria from their guts were isolated using standard procedures [70]. Individual females were collected from the cages in separate vials to prevent cross contamination and were placed in a freezer at -20^{o}C for 5 minutes to render them inactive. Individual females were surface sterilised with ethanol (70% - Bio-Strategy Laboratory Products, Australia) for 30 seconds. Sodium hypochlorite solution (0.25% - Coles Supermarket) was used to wash the external structures of the flies for 60 seconds. The flies

were then washed with distilled water three times to remove all contaminants externally and placed on clean filter paper.

Dissections were carried out under laminar airflow. Two pairs of sterilised forceps were used to dissect five female flies. Individual female Island flies were dissected aseptically in a clean Petri dish. A section in the ventral part of the abdomen was cut and teased open with the forceps. The mid and hind fly gut was carefully pulled out and removed from its body and placed on non-selective media, Yeast Extract Agar (YEA), in 90 mm Petri dishes. The obtained gut was carefully smeared on the agar surface and the Petri dish was closed and immediately sealed with parafilm. The media plate was labelled and kept in an incubator at 30^{o}C (± 1^{o}C) for 48-72 hours [47] and observed for growth. After two to three days of incubation the Petri dishes contained a mixture of various visually distinct bacteria colonies of different textures and colours. Isolates were obtained from all bacterial colonies for sub-culturing to pure isolates. As a control, YEA-only plates were also incubated with the streaked plates and observed for any growth.

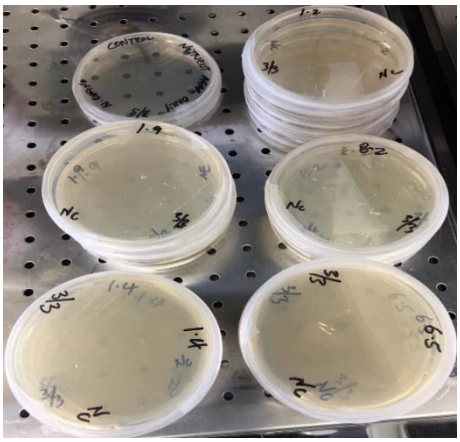
Figure 5: Streaked plates before Incubation

To obtain pure bacterial cultures by sub-culturing, under laminar airflow a single colony of visually distinct bacterial isolate was removed with an inoculation loop and streaked onto a fresh YEA petri dish (Figure 5). The Petri dish was again immediately closed and sealed with parafilm, labelled and placed in the incubator. It was again observed after 48-72 hours and a similar procedure carried out to isolate individual growing bacterial isolates [70]. Repeated sub-culturing (passaging) of the bacterial isolates produced pure bacterial cultures that were maintained on YEA plates (Figure 6). These pure bacterial cultures were renewed every 3 weeks to have fresh bacterial isolates available for use in assays. Pure bacterial isolates were preserved in glycerol at -80^{o}C for future reference.

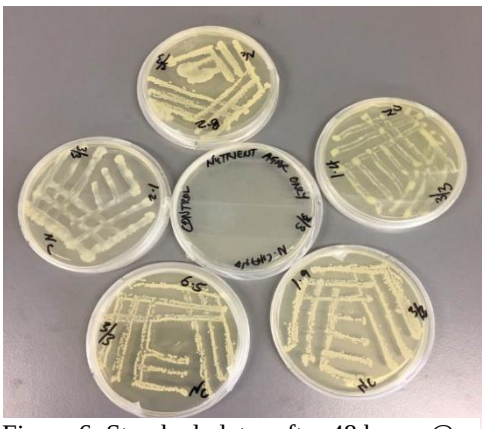
Figure 6: Streaked plates after 48 hours @ 30^{O}C

2.3 Island Fly Bacteria Identification

Island Fly bacterial identification was undertaken by the Australian Genome Research Facility (AGRF), Melbourne, Australia. Their 16S Sanger Sequencing service is a National Association of Testing Authorities (NATA), Australia; biological accredited service and the bacterial sequencing

employed universal bacterial primers to interrogate an approximate 800bp region of the 16S ribosomal RNA [71].

For the submission of bacterial samples to the laboratory, AGRF supplied 100 µL aliquots of PrepMan buffer (Thermo Fisher Scientific, Australia). Under laminar airflow, a small loopful of a single bacterial colony from a culture plate was obtained. The sample was suspended in 100 µl of the PrepMan buffer in a 1.5 ml micro centrifuge tube. It was vortexed for ~30 s at 1400 rpm, and then the sample was heated at 100°C in a water bath, for 5 minutes. The sample tube was dried with paper towel, labelled and sealed. The attenuated sample was submitted to AGRF's Melbourne laboratory for processing.

In the AGRF laboratory, the samples were subjected to an initial amplification using the universal 16S bacterial primers; amplification in a 20 µl reaction containing RNAse-free water, PCR buffer (Bioline, UK), Bioline Immolase DNA polymerase (Bioline, UK), 0.2 mM dNTPs (Bioline, UK), 2.5 mM $MgCl_2$ (Bioline, UK), 0.5 µM of forward primer (AGRF, Australia), 0.5 µM of reverse primer (AGRF, Australia), and 1:20 diluted sample.

The amplified product was visualised on a 2% agarose gel to confirm successful amplification. The amplified product was then subject to a manual purification employing magnetic beads and the purified product was re-suspended in HPLC grade water. A Big Dye Terminator (Thermo Fisher Scientific, Australia) sequencing reaction was performed for both the forward and reverse directions using the previously mentioned universal bacterial primers. The sequencing reaction was performed in a 15 µL reaction containing Big Dye Terminator v3.1 (Thermo Fisher Scientific, Australia), Big Dye buffer, 0.13 µM of forward primer (AGRF, Australia), 0.13 µM of reverse primer (AGRF, Australia) and purified PCR product.

The sequencing products were then subjected to an automated purification employing magnetic beads; the purified product was re-suspended in 0.5 M ethylenediaminetetraacetic acid (EDTA). Samples were then separated by capillary electrophoresis on an Applied Biosystems 3730xl Genetic Analyzer (96 capillary). Samples were blasted against AGRF's in-house 16S database using Paracel Blast. The database was manually collated from the Greengenes database [89].

Each 16S sequencing batch included a positive control sample and a negative control sample, thus each isolate was processed in triplicate. The positive control sample consistently generated the same BLAST results. There were no indications of sequences in the negative control samples, which would have required the entire batch to be reprocessed.

Data reports generated from the results enabled the isolates to be identified and included:

a) A raw chromatogram trace file.

b) A trimmed FASTA formatted text file. Reads were trimmed on the basis of the quality values assigned to the base calls.

c) A blast file comprising the top 20 hits against the in-house, AGRF curated 16S database.

d) A report summarising the top five hits from the BLAST file.

2.3 Headspace Collection

The headspace is the vapour portion in a vessel containing the sample (bacterial isolate), the dilution solvent (nutrient broth) and sometimes a matrix modifier. The volatile components in the headspace can be transferred onto an adsorbent material by using an air pump, to be chemically analysed for the separation of all volatile compounds contained in it. Different bacterial isolates were incubated in a nutrient broth solution.

All the apparatus used for the headspace collection including the screw caps with delivery ports and hoses were washed with odourless detergents (Abode Healthy Home Products, Australia) and hot water several times, then with acetone (99%, Sigma Aldrich, USA) and air dried for 24 hours prior to the next use.

13 g of standard nutrient broth ("Non-selective media for bacteria (General Media – 70123 (Sigma Aldrich, USA) was mixed with 1 litre of MilliQ water according to the supplier's direction. One hundred millilitres of the mixture was decanted into six, 250 ml Schott bottle and autoclaved at 121^{o}C for 15 minutes at 15 PSI. The autoclaved nutrient broth mixture was placed in laminar airflow to cool to room temperature. A loopful of a pure bacterial isolate was then obtained from the cultured plates and suspended in the broth. NaCl (20 g) was also added to the broth to remove excess water from cells and maintain healthy bacterial growth for optimum production of volatiles. The bottles were securely closed and sealed with parafilm, labelled and placed in a shaking incubator at 30^{o}C (±1^{o}C) at 150 rpm for 24 h of incubation. Uninoculated broth was included as a control in every replicate.

After the 24 h incubation period, the bottles containing the cultured bacteria were unscrewed and replaced with sterilised screw caps having a two-hose connection port (Duran, Germany; Screw Cap GL 45, PP, 2 Port GL 14) attached with delivery tubes (Figure 7). This cap exchange process was immediately conducted over the Schott bottles whilst still in the shaking incubator. The delivery tube included one hose to "push" air into the Schott bottles from an air delivery system (0.05 l min^{-1}

Figure 7: Schott bottle delivery system with TD tube

air with charcoal filter (ARS, USA)), which would drive the bacterial headspace volatiles produced over the 24 h period to be pushed out through the other tube connected to a Thermal Desorption (TD) tube containing Tenax A adsorbent [72] (Sigma Aldrich, Tenax A Mesh 60/80, Fritted 89 mm glass tube). The TD tubes had previously been conditioned at 300^{o}C for 30 minutes with nitrogen gas flow at ~0.05 l min^{-1}. The tubes were immediately sealed and wrapped in aluminium foil, only to be opened immediately prior to analysis. The Schott bottles were recapped with normal screw caps.

All the fittings in the collection apparatus were "gas tight" to prevent any contamination or loss of pressure. The headspace volatile collection was undertaken for six minutes, which provided at least three full displacements of the available headspace in the Schott bottles (approximately 150 ml of headspace in a 250 ml Schott bottle having 100 ml of broth with isolates). After collection, the TD tubes were detached, sealed with screw caps and completely wrapped in aluminium foil for storage for later analysis by GC-MS. The batch numbers on the TD tubes were recorded in accordance with the corresponding bacterial isolate or broth.

All the bacterial isolates were autoclaved at 121^{o}C for 15 minutes at 15 PSI for disposal after headspace collections.

2.4 Gas Chromatography Mass Spectrometry (GC-MS) Analysis

Total-ion chromatograms and mass spectra were recorded on a Shimadzu GC-MS-QP2010 (Shimadzu Corporation, Japan) instrument [73] using a Restek Rtx-5Sil column (30 m × 0.25 mm I.D. × 0.25 μm film thickness) and helium (BOC, North Ryde, Sydney) (99.999 %) as a carrier gas. For the temperature program, the initial column temperature was set to 40°C and held for 2 minutes before it was increased to 85°C by 10°C/minute, held for 0.5 minutes, then increased by 5°C/minute to 120°C before ramping by 10°C/minute to 300°C where the final temperature was held for 2 minutes. The injector temperature was set to 270°C and the detector to 290°C. Mass spectra were recorded in EI mode (70 eV), scanning from 40 to 620 *m/z*. The TD tubes with bacterial isolate headspace collections were placed on the TD autosampler and the GC-MS program was run to conduct the analysis.

Mass spectra were analysed using the Lab Solutions GC-MS solution software version 2.40. The retention times (Appendix 2) and area under the peaks were recorded for the peaks emitted by the bacterial isolates and the broth, which served as a control. For each peak, the fragmentation pattern was compared to the internal libraries (NIST21 and NIST107) to tentatively identify the collected volatiles.

2.5 Y-Tube Olfactometer

The Y-tube olfactometer provides a binary choice test between a stimulus and control or comparisons between two stimuli. The Y-tube olfactometer consisted of three clear glass tubes (70 mm internal diameter) of equal length (130 mm) that was joined as in a Y shape (Figure 8). The upper two arms, known as choice arms, were joined together at any angle of 75^{o} intersection with an angle of 142.5^{o} left between the base arm and the choice arms. The two choice arms were connected to arms (150 mm) by airtight male-female joints overlapping 20 mm. The tubular arms had wire gauze separators in them (70 mm) to prevent the flies from accessing the stimuli directly upon reaching the end of the choice arm. The stimuli were placed in the middle of the tubular arm and the end of the tubular arm had rubber bung stoppers with hose connections to allow for inflow of air from the air delivery system (Figure 8).

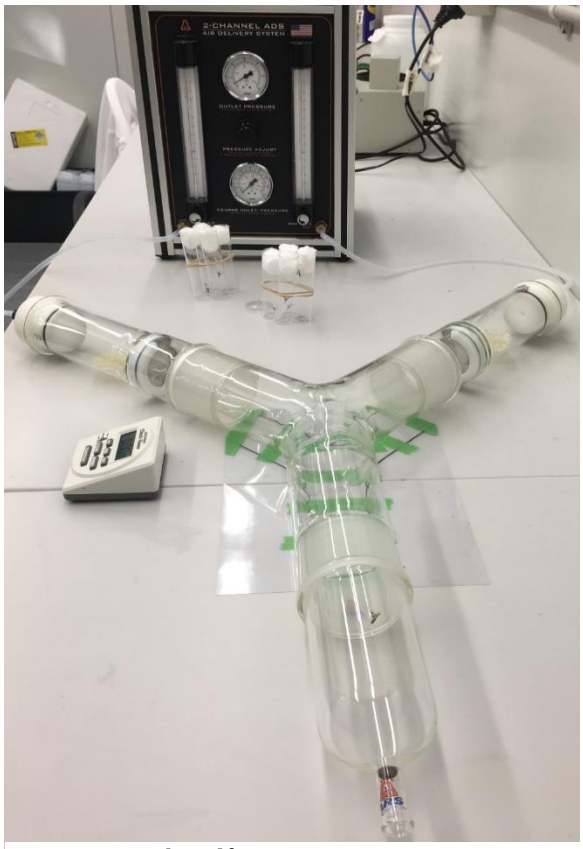

Figure 8: Y-Tube Olfactometer setup

All components of the glass Y tube olfactometer were thoroughly cleaned with warm water and odourless detergent (Abode Healthy Home Products, Australia) and rinsed six times with hot water and then acetone (99% - Sigma Aldrich, USA) to remove any contaminants and all components were oven dried at 200^{o}C overnight. Island Flies 10-14 days old, both males and females that were provided with water but unfed for 24 hours, were used in this assay. Six males and six females were used to test responses to each bacterial isolate versus agar as a control [74]. The bacterial isolates were placed in 30 mm Petri dishes with YEA media, sealed, labelled and incubated for 24 hours at 30^{o}C (+1^{o}C). Petri dishes of YEA only as control were also prepared for the assays. The Y-tube olfactometer was connected to a push system air delivery apparatus (ARS, USA) and the airflow was adjusted to 0.5 l min$^{-1.}$ Delivered air was passed through a charcoal filter to clean and then a bubbler for humidification. The assay was conducted in a room that was separate from the Island Fly rearing rooms and had similar lighting conditions as the rearing room and temperature of 23^{o}C (±2^{o}C). A "ladder" was placed under the Y tube olfactometer indicative of the distances the flies were travelling.

Individual flies were placed in the release chamber located at the bottom of the base arm of the olfactometer and allowed to settle down for two minutes. A 30 mm Petri dish containing a bacterial isolate was placed in one choice arm of the olfactometer, while a 30 mm Petri dish with only agar

was placed in the other choice arm. The air system was then switched on and adjusted to the required flow rate. As soon as the flies entered the base arm of the olfactometer from the release chamber, timing was started for the assay and observations were made on the movement of the fly. The 'decision time' was recorded when the fly started to move into one of the Y-arms of the olfactometer past the joining point of the Y-arms. When the fly reached the midpoint of the choice arm of the olfactometer, this time was again recorded as 'residence time'. Observations were made up until 120 seconds to observe if the fly continued to move towards the far end of the choice arm, and closer to the stimulus. The stimulus was regarded as very attractive when the fly moved rapidly to the end of the choice arm and remained there until completion of the assay. A stimulus was regarded as moderately attractive when the fly remained mainly in the residence area and did not move toward the end of the Y-arm. The stimulus was regarded as non-responsive when the flies moved to the Y-arm containing agar only (control). Flies that did not respond to the stimuli or control within the 120 seconds allocated time were regarded as non-responsive and were discarded from the assays. Each fly was used in the assay only once and discarded from the project after being exposed to an isolate.

Once an observation with a fly was finished, the arms of the olfactometer were swapped for randomisation and to prevent any inadvertent positional effects and fresh plates of bacterial isolates and agar were used for further assays. The male and female flies were released alternately to allow for randomisation as well. This procedure was replicated for six males and six female flies for the five bacterial isolates and control. The airflow was checked for each assay to ensure the correct flow rate was maintained. When a particular isolate assay was finished, the whole set up was dismantled and cleaned prior to reuse. The apparatus was oven dried (200^{o}C overnight) before setting it up once again to be used for a different bacterial isolate assay. All Petri dishes with isolates and agar were disposed of after assays of the isolates were completed.

2.5.1 Data analysis method

The data of each sex of Y-tube experiments were separately analysed by using a linear model, followed by posthoc analysis to see if there were significant differences in fly responses between bacteria. R-3.4.2 (R core team 2017) was used for all analyses.

2.6 Wind Tunnel

For the wind tunnel assays, an Insect Bioassay Wind Tunnel (Analytical Research Systems, USA; Model# OLFM-WT-12X12X48) (Figure 9) constructed of clear heavy wall acrylic plastic framed in an aluminium structural system with dimensions of 12”×12”×48” (30.48 cm×30.48 cm×121.92 cm) with 4” (10.16 cm) exhaust duct was used. It was thoroughly wiped clean with ethanol (70% - Bio-Strategy Laboratory Products, Australia) and allowed to run with full air flow for at least 30 minutes to remove any volatiles *via* the exhaust ducts prior to each days’ assay being conducted. Six males and six females per bacterial isolate and agar were used for this assay. The flies were provided with water and not fed for 24 hours prior to the assays. The airflow system was connected to a glass chamber that had the bacterial isolate in 30 mm Petri dishes and the flow was adjusted to 0.5 L min^{-1}.

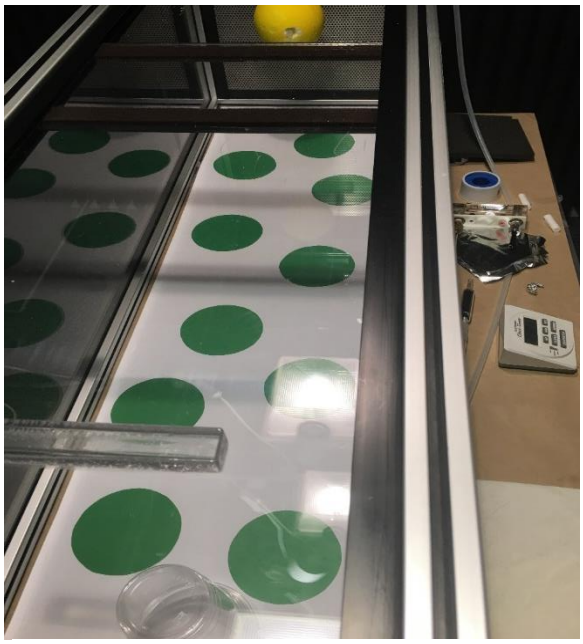

Figure 9: Wind Tunnel setup in laboratory

The flies were released as separate groups of six males and six females in the tunnel and allowed two minutes to settle down. The air flow system was switched on to carry the bacterial isolate olfaction (odour plume) into the wind tunnel and blow it downwards towards the released flies [75]. Green circles were placed beneath the wind tunnel chamber to simulate vegetation. The time was noted when the released flies crossed the one metre mark in the wind tunnel from their release point, while moving in response towards the stimuli. An artificial yellow styrofoam ball (8 cm diameter), simulating the size of a normal orange fruit, was hung inside the tunnel opposite of the fly release point to serve as a visual cue. An odour plume was released from an inlet tube that was connected through the ball; being defined as the odour release-point.

After a bacterial isolate assay was completed, all flies used in the assays were discarded from the project to be used in the rearing activity. The wind tunnel was wiped clean with ethanol (70% - Bio-Strategy Laboratory Products, Australia) and allowed to purge for 30 minutes prior to introducing the next bacterial isolate. This procedure was replicated for six males and six female fly groups for the five bacterial isolates and control.

2.6.1 Data analysis method

The data of each sex of wind tunnel experiments were separately analysed by using a linear model, followed by posthoc analysis to see if there were significant differences in fly responses between bacteria. R-3.4.2 (R core team 2017) was used for all analyses.

2.7 Electroantennography (EAG)

Electroantennography (EAG) records small voltage fluctuations between the tip and base of an insect antenna during stimulation with volatile compounds. The measured voltage fluctuation is caused by electrical depolarisations of olfactory neurons in the insect's antenna. EAG assays were performed to investigate the response of the Island Fly towards the volatile stimuli produced by its gut bacterial isolates.

The EAG apparatus (Manipulator Assembly, Type MP-22, Syntech, Germany) was set up as in Figure 10. Capillary tubes were used to prepare micropipettes for use in the assays by pulling them under high heat in a vertical micropipette puller (Cat. 2001, Scientific Research Instruments, England). The micropipettes were filled with phosphate-buffered saline (PBS) using a syringe and care was taken while filling the pipettes to avoid the formation of any air bubbles in the micropipettes as they would block the electrical signals. The filled micropipette was fixed onto the "different" electrode holder ensuring that the silver wire (0.3-0.5 mm length) was properly in place inside the micropipette.

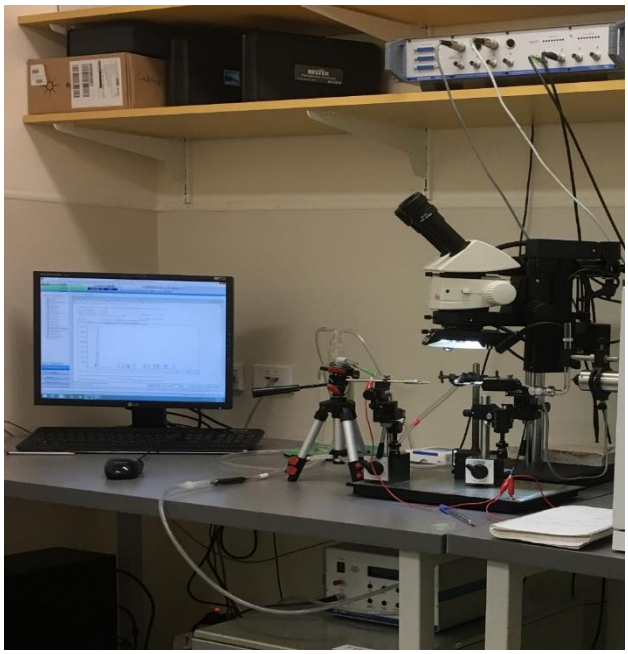

Figure 10: EAG setup in laboratory

The other micropipette was used for fixing the Island Fly head. Island Flies that were 10-14 days old and unfed for 24 hours were used for this assay. An individual fly was placed in a freezer at -20ºC for two to three minutes to stun it and make it easier to work with. The stunned fly was placed under a microscope and micro-surgical scissors were used to separate the head. The body of the fly was discarded.

The head of the Island Fly was carefully mounted onto the PBS filled micropipette under a stereo microscope [76]. Care was taken not to damage any structures, especially the antennae, on the head of the Island Fly or to prevent inserting the tip of the micropipette too deeply into the head. Once a firm mounting was achieved, the micropipette with the attached head was fixed onto the "indifferent"

electrode holder of the EAG apparatus. The tip of the antennae was positioned very close to the tip of the open micropipette. The tip of the antennae was slipped very carefully into the open tip of the recording micropipette using the fine controls of the manipulators. The micropipettes were carefully inspected for absence of air bubbles. Then the computer program was started and a relatively stable baseline indicated the antennae had made proper contact with the electrodes.

The stimuli were prepared in laminar air flow, by placing one mL of the bacterial isolate solution onto pre-folded filter paper 1.5 cm^2 and allowed to air dry. The same was done to prepare the broth stimuli for control assays and another Pasteur pipette was prepared with only filter paper as a no odour control. The filter paper with the stimulus was placed inside a Pasteur pipette and pushed mid-way through its length. The setup was connected as in Figure 11.

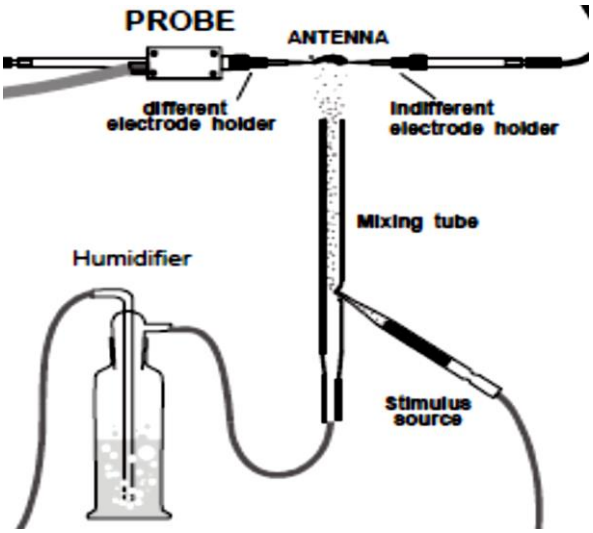

Figure 11: EAG setup with connected antenna
Source: EAG Manual, Syntech

Humidified air was used as the carrier for the compound vapours and controlled by a Syntech Stimulus Controller CS-55. The EAG signal was passed through a Syntech IDAC 4 and analysed using GC-EAD 2010 software version 1.2.2 [77].

Whilst recording the electrical signals on the computer, firstly an air-only pulse was recorded as a baseline signal. Then the Pasteur pipette with bacterial isolate stimuli was fixed on to the mixing tube and puffed once by pressing the pedal. Another two automatic puffs were generated by the Stimulus Controller 10 seconds apart. The voltage difference was recorded and the antenna was allowed to rest for one minute. The pedal was again pressed for another round of stimulus after the one-minute interval. Each bacterial isolate assay was replicated three times, then an air-only puff was conducted to complete the assay.

The mounted Island Fly head remained active and provided steady signals for 30 minutes only. After 30 minutes the signal declined and the assay was terminated. Both the micropipettes were drained of the used PBS and rinsed, then filled up with fresh PBS to be used for next bacterial isolate assay.

CHAPTER 3: RESULTS & DISCUSSION

3.1 Island Fly Rearing

The rearing techniques of Island Fly has not been published yet by any researchers although a number of them have tried in the recent past to establish colonies. These Australian fruit fly researchers (Morrow [14], Riegler [14], Schutze [15], Jessup [16], Royer [17] and Lee [18]) found female Island Flies produced low numbers of eggs (approximately 10-15% of Qfly's egging rate), making colony rearing extremely difficult. The only place where a colony has been successfully reared up to eight generations in the laboratory was in SARDI, South Australia. Following discussions with the researchers in SARDI and identifying their challenges and successes, conditions were optimised to allow the successful establishment of Island Flies at Macquarie University. In the six months period of rearing, three generations of island Flies were successfully reared for use in this project, but this could be improved with better rearing conditions as mentioned below. The factors considered and procedural modifications made to improve the SARDI procedures are discussed in the next sections.

3.1.1 Seasonal Patterns

Island Flies in the Macquarie University laboratories were observed to lay eggs (approximately 1 to 1.5 g) and reproduce normally during the warmer months (February to May). As temperatures decreased towards the middle of the year (June to August), a gradual decline was noted in their laying pattern and then the flies stopped laying altogether. Although being maintained in a controlled environment room, the Island Flies were observed to copulate less and produce non-viable eggs. The lower copulation and egg production may be due to their reaction naturally towards non-availability of suitable hosts and their fruits for oviposition during the winter months. This behaviour most likely corresponds to their inherent natural seasonal pattern behaviour and may be ovarian diapause or reproductive arrest as noted in the study of *Drosophila melanogaster*, [78] which has similar food preferences as Island Fly.

3.1.2 Temperature

It was noted that the Island Flies were reared in a large laboratory room in SARDI that did not have any temperature regulation mechanisms apart from a standard air conditioning unit. Temperature plays a very crucial role in the life cycle developments of all fruit flies. A "degree-day" (phenology) modelling study of Island Fly [4] conducted at SARDI in 2011 suggested that temperatures between 26-32^{o}C were ideal for Island Fly's optimum development and reproduction. Temperatures between 15-26^{o}C slowed down the developmental life cycle significantly and when lower than 15^{o}C the development was stalled [4]. Temperatures above 32^{o}C became too warm for the Island Fly and they dehydrated very rapidly and had very low survival rates. Although, the Island Flies for this project

were reared in a controlled environmental room, the data loggers placed in the room at different intervals indicated that the temperature dropped below the 27^{o}C set point, especially during night phases. The occasional technical failures to the operating system in our projects' Island Fly's rearing rooms could have contributed to slower development in rearing the flies.

3.1.3 Lighting

Lighting regimes, intensity and the photoperiod are other factors that play vital roles in the rearing of Island Flies in laboratories. In the SARDI laboratories, all the lighting needs were provided by the natural photoperiod cycles of the sun and moon as all the rearing cages were placed alongside the windows, conducive with their circadian rhythm. This allowed the flies in the SARDI labs to reproduce normally and an increase in population numbers was noted. In the Macquarie University rearing room, the lights were initially adjusted to accommodate for the optimum requirements of Island Fly's growth and development as there was a complete absence of natural light. However, due to unforeseen technical issues, the lights reverted to the default settings on numerous occasions and this may have adversely affected their reproductions. The F0 generation were trying to adapt to the new laboratory environment during the establishment phase and the light inconsistency may have affected the throughput required for a robust population in the subsequent generation. The low reproduction numbers did not allow for many experimental replications to be conducted and produced statistically non-significant results.

3.1.4 Diet Modification

The rearing of Island Fly was modified in the egg laying and larval stages from the SARDI procedures. In SARDI, the eggs were manually collected after being laid in artificial oviposition devices and washed with water. The eggs were seeded into liquid larval diets that consisted primarily of orange juice, yeast and antibiotics, but at Macquarie University the female flies laid eggs directly into the gel diet. The larval gel diets were prepared according to Chang's [66] and Tahereh's [67] gel diet formulations. The diets had slight modifications in terms of ingredients being procured from different suppliers. Larval gel diets were filled into diet containers that served as oviposition devices for the female flies. The eggs laid in the larval gel diet hatched within the diet with the larvae having a readily available food source. The larvae developed with the gel diet until they were ready to "jump out" for pupation. Tahereh *et. al.* also noted that gel diets consisting of agar were more shelf stable in comparison to liquid formulated diets [67] and they lead to improved productivity and adult fly quality. This revised procedure of directly seeding the eggs in larval diets avoided the additional tasks of preparing liquid larval diets and oviposition devices. The manually harvested eggs (approximately 1-1.5 g) needed to be seeded rapidly, usually within 4-6 hours of laying, to maintain a high viability and needed delicate handling. The revised procedure of using gel diets, directly seeding of eggs in

the gel diet and avoiding manual handing of the eggs were improvements that may contribute to rearing and establishment of Island Flies in laboratories.

3.1.5 Wheat Germ Oil Addition

The diet of Island Flies raised in SARDI contained the ingredients as detailed in the diet formulation in Appendix 2. In our larval diets, organic wheat germ oil (Melrose Health Group) [13] was additionally added to all batches to assist in the development and hatchability of Island Flies [67]. As reported by Chang and Vargas, increases in egg production, egg hatchability, pupal recovery and greater percentage of adult fliers [68] are observed for larvae reared with wheat germ oil in diets compared with those reared on the diet without wheat germ oil. Wheat germ oil is believed to assist in the advancement of colonies in the early phases of development due to being a good source of protein and vitamin E for the flies [90].

3.1.6 Dark Incubation Environment

The larval diet seeded with eggs by the female flies were placed in a dark environment under black storage crates, without any lights, to simulate the environment inside the fruit tissues where the eggs are naturally laid by the female flies. The simulation of the dark phase prompted the Island Fly eggs to develop at normal rates and contributed to the colony establishment. Also, there was good moisture retention by the vermiculite layer in the rearing cages under the black storage crates, while allowing adequate gaseous exchange to take place. This procedure was different from that of SARDI and was conducted to provide an environment as close to nature as possible to assist Island Fly's laboratory establishment.

3.1.7 Summary

The following procedures were used in our project in an attempt to rear and establish Island Flies for a continuous laboratory colony throughout the year:

a) Using a "purpose built" controlled environment room for fruit fly rearing with regulation for temperature, light and humidity

b) Using a gel based larval diet instead of liquid formulations

c) Using organic wheat germ oil in larval diets

d) Female flies laying directly into the larval gel diets

e) Combining the oviposition devices to serve as diet containers for larval rearing

f) Using a dark incubation environment.

3.2 Bacteria Identification

Using 16S Sanger Sequencing analysis (AGRF, Melbourne), five species of Island Fly gut bacteria associated with female Island Flies were identified *viz. Bacillus sp*., *Citrobacter freundii*, *Bacillus subtilis, Providencia rettgeri* and *Klebsiella oxytoca* (Table 1). *Bacillus sp*. of bacteria may be further identified by increasing the base pair match ups to species level (*B. cereus* or *B. anthracis* or *B. thuringiensis*), however the AGRF analysis was limited to 800 base pairs only and time limitation in this project did not allow for this. The bacterial isolates in this project were only derived from female flies, but there is a recent publication by Gujjar *et al.* [79] that *Bactrocera dorsalis* and *Zeugodacus cucurbitae* male fruit flies have slightly different cohorts of bacteria biomes to female fruit flies. Some of the species of bacteria identified in this project are the same as those obtained in other species of fruit flies from their gut microbiomes [23, 33, 41, 70, 79]. This suggests that these bacterial species are commonly associated with fruit fly microbiomes.

Project ID code	Sequence Entry	Hit Length	% Identity	E value
1.2	802687 *Bacillus sp.* str. B4RO09 HQ015742.1 1..1412	724	100	0.0
1.4	736448 *Citrobacter freundii* str. MRB070408-2 GU126683.1 1..1434	711	100	0.0
6.5	725329 *Bacillus subtilis* str. Bio AAF1 FJ966222.1 4..1427	720	100	0.0
CO 1	113218 *Klebsiella oxytoca* str. SB136=ATCC 49131 AJ871857.1 1..1454	711	100	0.0
CO 3	128082 *Providencia rettgeri* str. DSM 4542 AM040492.1 1..1497	708	99.718	0.0

The Expect value (E) is a parameter that describes the number of hits one can "expect" to see by chance when searching a database of a particular size. The lower the E-value, or the closer it is to zero, the more "significant" the match is.

Table 1: Bacteria identified from the gut of female Island Fly

Bacillus subtilis is found naturally in soil and vegetation. Its cells are rod-shaped and is a Gram-positive bacterium. It does well in temperatures of 25-35^{0}C and is also capable of nitrate ammonification as well as butanediol fermentation. Its main habitat is the soil environments and undergrowth of plants. Taxonomically it belongs to Bacteria domain: Firmicutes phylum:, class: *Bacilli*, order: *Bacillales*, family: *Bacillaceae*, genus: *Bacillus* and species *subtilis* [98]. *B. subtilis* bacteria are used on plants as a fungicide and are non-pathogenic. At temperatures of 37^{0}C and a pH of 8 its antibiotic optimal activity occurs. They also secrete enzymes, "such as protease, xylanase, amylase, lipase, pullulanase and chitinase [72, 97].

Citrobacter species of bacteria that includes *Citrobacter freundii*, are long rod-shaped bacteria typically 1-5 μm in length and aerobic gram-negative bacilli. Its habitat includes the environment (water, soil and sewage), the intestinal tracts of animals and humans and food. Taxonomically it belongs to Bacteria domain: phylum *Proteobacteria*, class: *Gammaproteobacteria*, order: *Enterobacteriales*, family: *Enterobacteriaceae*, genus: *Citrobacter* and species *freundii* [98]. *C. freundii* cells have many flagella used to move about, but a few are non-motile. It belongs to the family of *Enterobacteriaceae*. *C. freundii* produces enzymes phosphatase and is responsible for reducing nitrate to nitrite in the environment for the nitrogen cycle [72, 97].

Klebsiella oxytoca is a cylindrical rod-shaped bacterium measuring 2 μm by 5μm and it is indole-positive being able to grow on melezitose, but not 3-hydroxybutyrate. It is a gram-negative facultative anaerobe bacterium that can hydrolyze cellulose and fix nitrogen. *K. oxytoca* can be found in a wide range of places and referred to as ubiquitous and opportunistic in nature. Taxonomically it belongs to Bacteria domain: phylum *Proteobacteria*, class: *Gammaproteobacteria*, order: *Enterobacteriales*, family: *Enterobacteriaceae*, genus: *Klebsiella* and species *oxytoca* [98]. It is known to breakdown cyanide especially from wastewater systems and when added to post-irradiation diet significantly improves the performance of sterile male Med fly [72, 97].

Providencia rettgeri is found commonly in water-ways and land environments and is a Gram-negative bacterium. It has the ability to produce acid from mannitol and can be incubated at 37^{o}C in nutrient broth or agar. *P. rettgeri* does not ferment lactose and does not produce acid from xylose. It does not produce gas from glucose and also does not produce hydrogen sulphide. Taxonomically it belongs to

Bacteria domain: phylum *Proteobacteria*, class: *Gammaproteobacteria*, order: *Enterobacteriales*, family: *Enterobacteriaceae*, genus: *Providencia* and species *rettgeri* [98]. It been isolated from field captured *Drosophila melanogaster's* haemolymph [72, 97].

The association of the bacteria from this study has been reported previously by many researchers. The midgut and crop of *B. cacuminata* had dominant species of *K. oxytoca, C. freundii* and *Enterobacter cloacae* when extracted and grown on nutrient agar. *Enterobacteriaceae* were dominant mainly in the midgut region while *Firmicutes* were mainly occurring in the crop regions [26, 97]. Robacker *et. al.* (1997) reported that *C. freundii* was cultured in tryptic soy broth and its headspace had greater amounts of several pyrazines, 1-pyrroline and ammonia [91, 95]. The assays conducted by Yuval *et. al* (2008) stated that the most prominent bacterial species in Med fly were *Enterobacter spp., C. freundii, Klebsiella spp, Pectobacterium* and *Providencia stuartii* [35]. Furthermore, Wang *et. al* (2014) found the major genera of *Enterobacter, Serratia, Klebsiella* and *Citrobacter* on *Bactrocera minax* [46].

Morrow *et. al.* (2015) noted Island Fly to mostly have *Acetobacteraceae,* particularly *Acetobacter* in their gut microbiome [11]; however their result was based on a single Island Fly sample that was used in the assay. They concluded that the gut microbiome of Island Fly was closely related to the gut biomes of *Drosophila*, as both the species were attracted to over-ripe or rotting fruits and acquired similar microorganisms from their ecologically different environment to other species. Island Fly microbiome was distinctly different to the gut microbiomes of other species of *Bactrocera* and Medfly in their project.

Gujjar *et al.* [79] reported that *B. dorsalis* female flies had *C. freundii* as their gut microbiome together with other *Enterobacter* species and the same was found in this project. However, *B. dorsalis* males had *P. rettgeri* and *K. oxytoca* as their gut microbiome composition and *Zeugodacus cucurbitae* males had *B. subtilis* bacteria. Gujjar *et al.* strongly suggested that future fruit fly management techniques should take into consideration gender specific gut bacterial colonies.

3.3 Y-Tubes Olfactometer Assays

The volatiles obtained after 24 hours from the bacterial isolates grown on yeast extract agar were used for Y-tube olfactometer assays. Island Flies were individually exposed to the volatiles directly emitted by the incubated bacteria and then examined for their responsiveness to these volatiles from the bacteria isolates, as described in Section 2.5. Generally, female flies were attracted and were observed for faster wing movements (similar to dancing) and ovipositor extrusions when moving towards all the bacterial isolates while males did not show this trend. On average, females were faster but not

statistically significant in making a decision for *Bacillus sp.* and rapidly moved into residence phase, suggesting strong attraction towards it.

3.3.1 Summary of Y-tube Experiments

Y-tube experiments were conducted as described in Section 2.5. The Y-tube experiment results in Table 2 showed that most of the Island Flies were attracted towards *Bacillus sp.* bacterial isolate (67%) while only 33% were attracted towards the control (agar). Least number of flies were attracted towards *C. freundii* (33%) while most flies opted for the control (agar) in the 2-choice tests.

	Bacillus sp.	*C. freundii*	*B. subtilis*	*K. oxytoca*	*P. rettgeri*
Bacterial Isolate	67	33	50	58	42
Control (Agar)	33	67	50	42	58

Table 2: Percentage of Island Flies attracted to bacterial isolate over control

Figure 12 shows a summary of the data of decision time for both sexes in Y-tube experiments. Comparison in decision time between sexes showed there was no statistical significant difference, due to low sample sizes used and low replications; between sexes across all the bacteria, as shown in Figure 12 with p values all > 0.05. In females, the type of bacteria was not a statistical significant factor, due to low sample sizes used and low replications; for decision time in the Y-tube experiments ($F(1, 25) = 1.555$, $p = 0.217$). In males, the type of bacteria was a marginal factor for decision time ($F(4, 25) = 3.271$, $p = 0.027$). However, pairwise comparison between groups showed that decision time between groups were not statistically different ($p > 0.05$).

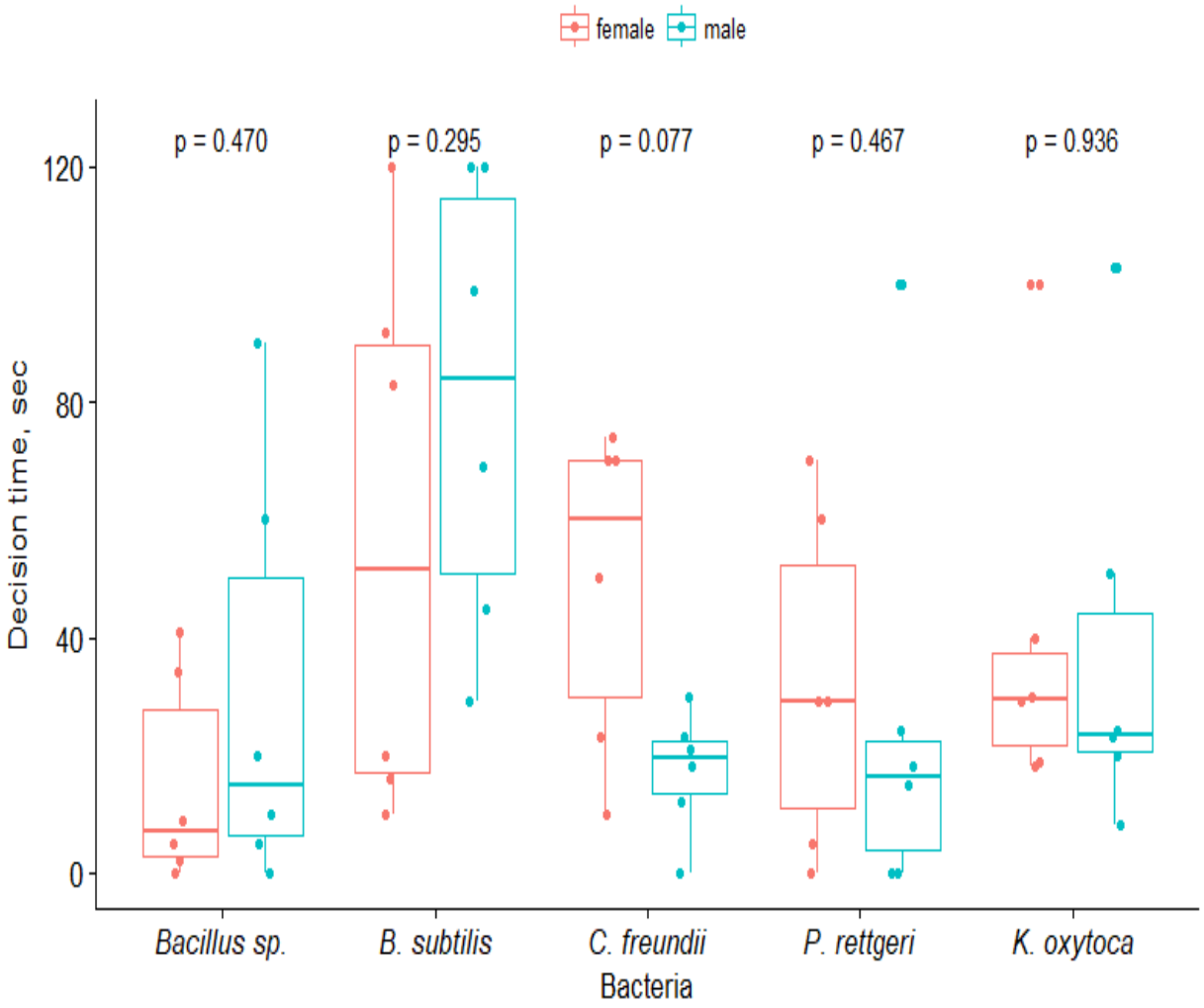

Figure 12: Summary of decision time in Y-tube experiments. P-values indicate there were no differences in decision time between sexes for all bacteria.

Figure 13 shows a summary of the data of residence time for both sexes in the Y-tube experiments. There was no significant difference found in residence time between sexes across all the bacteria ($p > 0.05$). Type of bacteria was not a significant factor for residence time in the Y-tube experiment for both females ($F(4, 25) = 2.372$, $p = 0.080$) and males ($F(4, 25) = 0.596$, $p = 0.670$).

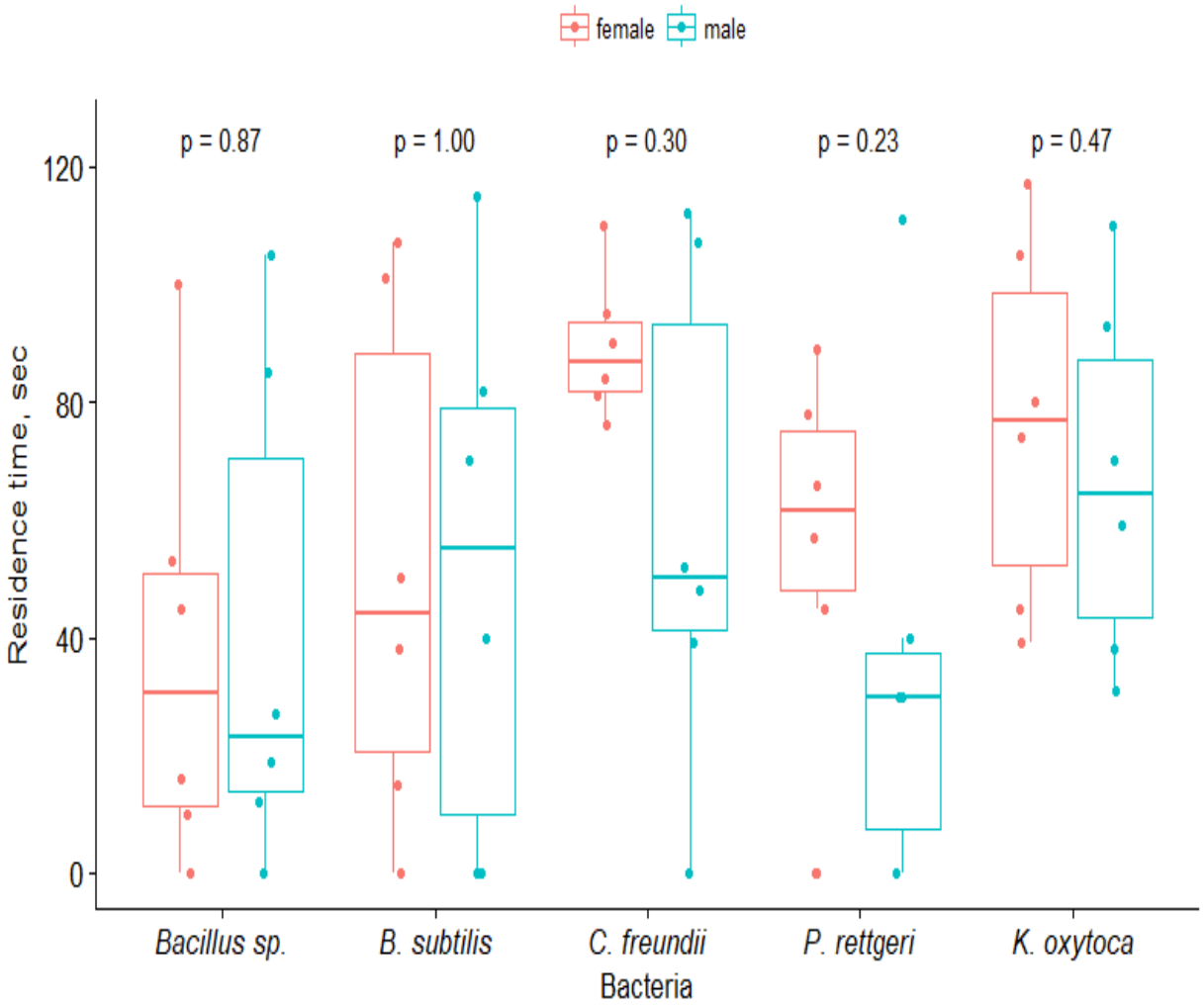

Figure 13: Summary of residence time in Y-tube experiments. P-values show there were no differences in residence time between sexes.

Male Island Flies did not show any significant differences amongst them for bacterial isolates. On average the males made a rapid decision with *C. freundii* and observations showed them being deterred from its bacterial volatiles and moving towards the control.

Observation during the assay showed differences in responses, from rapid to moderate to no attraction between all the isolates. This suggested that all the isolates had varying levels of effects on the males and females of the Island Fly for decision time and residence time. Although the results were not statistically significant due to low sample sizes used and low replications, on average the Island Flies made a decision to move towards *Bacillus sp.* in the shortest time, while Island Flies took the longest decision time for *C. freundii*. Once the Island Flies had made a decision, they then proceeded into residence in a similar manner. On average, *Bacillus* sp. showed the quickest residence time due to its attractiveness and *C. freundii* had the slowest time on average but but all results were not statistically different due to low sample sizes used and low replications.

3.4 Summary of wind tunnel experiments

Wind tunnel experiments were conducted as described in Section 2.6. Bacteria volatiles were released as an odour plume into a wind tunnel with wind flow from one end of the tunnel and Island Flies released from the other end in separate groups of females and males. Observations were made for two minutes on the responses of the Island Flies towards the odour plume and the time taken for them to cross a one metre mark on the wind tunnel from their release point. The results in Table 3 showed that female and male island flies took the shortest time of 70.3 and 72.0 seconds respectively to cross the one metre mark for *Bacillus sp*. Male and female Island Flies took the longest time of 107.3 and 106.5 seconds respectively to cross the one metre mark for *C. freundii* isolate.

Bacterial Isolate	*Bacillus sp.*	*C. freundii*	*B. subtilis*	*K. oxytoca*	*P. rettgeri*
Female	70.3	106.5	104.3	97.7	93.0
Male	72.0	107.3	105.2	99.5	102.7

Table 3: Time (seconds) for Island Flies to cross one metre mark from release point in the Wind Tunnel experiment

Figure 14 shows a summary of the time data of crossing the one metre mark for both sexes in wind tunnel experiments.

There was no significant difference found in cross time between sexes of Island Fly in all groups ($p > 0.05$), as shown in Figure 3. The type of bacteria isolate was a significant factor for cross time in wind tunnel experiments for both females ($F(4, 25) = 5.575$, $p = 0.002$) and males ($F(4, 25) = 5.524$, $p = 0.003$).

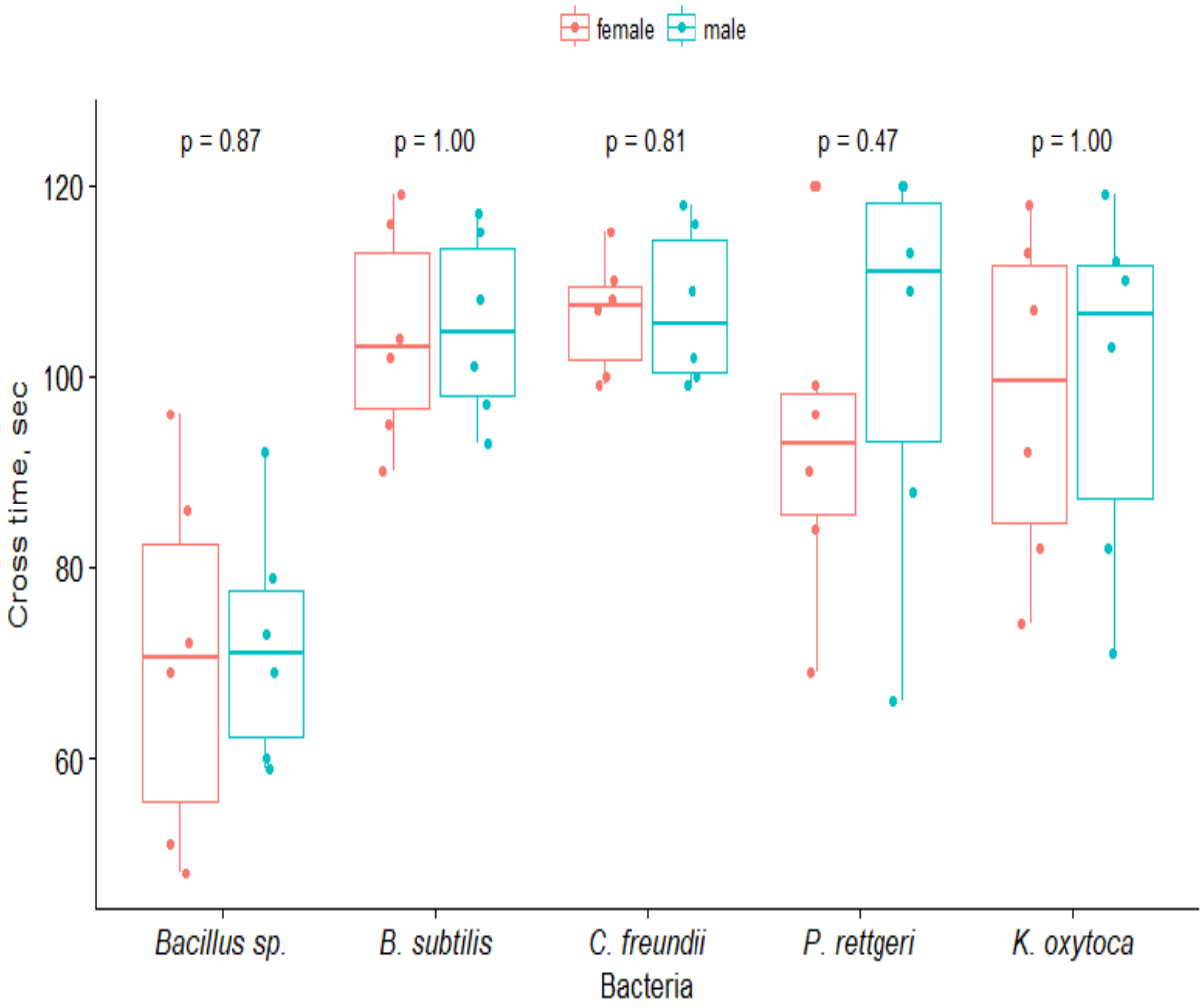

Figure 14: Summary of wind tunnel experiments. P-values show there were no differences in cross time between sexes in the testing bacteria.

The result of pairwise comparison between female groups showed that the observed cross time of *Bacillus sp.* was significantly shorter than *B. subtilis* and *C. freundii*, but not statistically different from that of *P. rettgeri* and *K. oxytoca* (Figure 15).

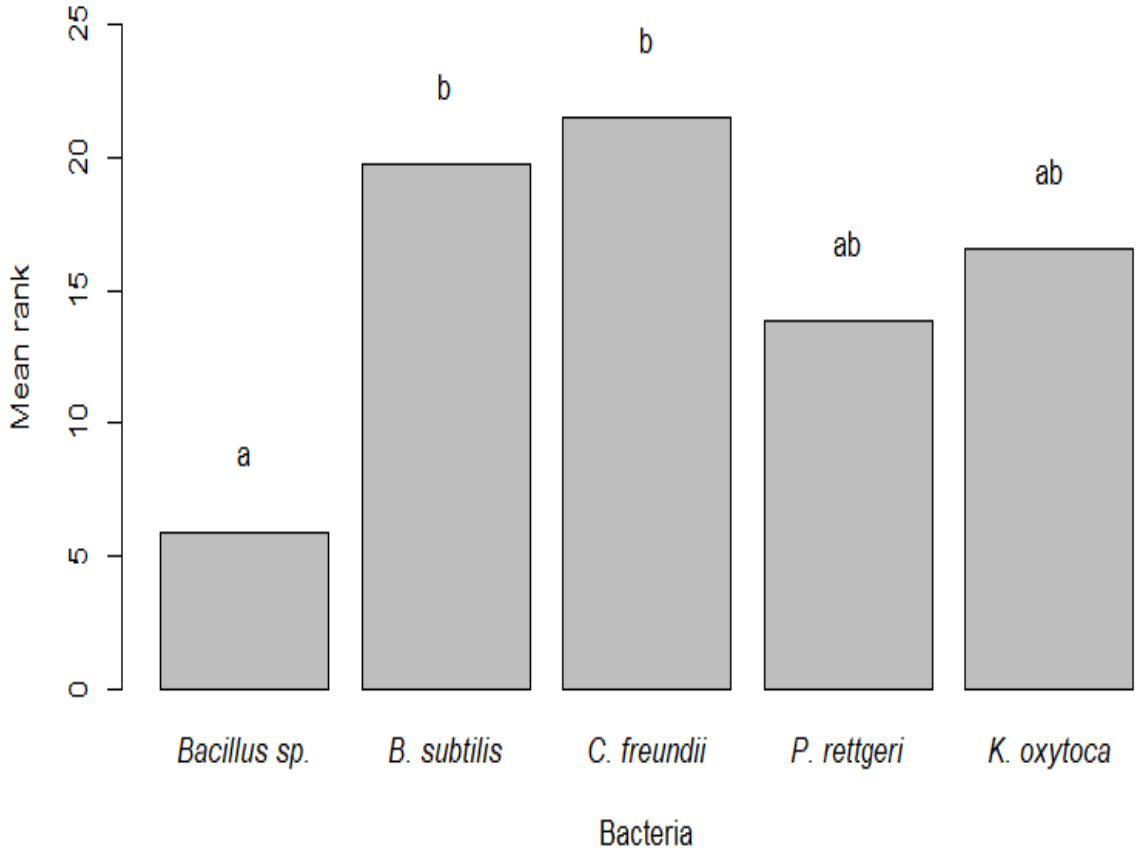

Figure 15: Female mean rank in cross time in wind tunnel experiment. Same letters indicate no significant differences between groups.

The results between Island Fly male groups showed that the observed cross time of *Bacillus sp* was shorter than that of *C. freundii* and *P. rettgeri*, but not different from that of *B. subtilis* and *K. oxytoca* (Figure 16).

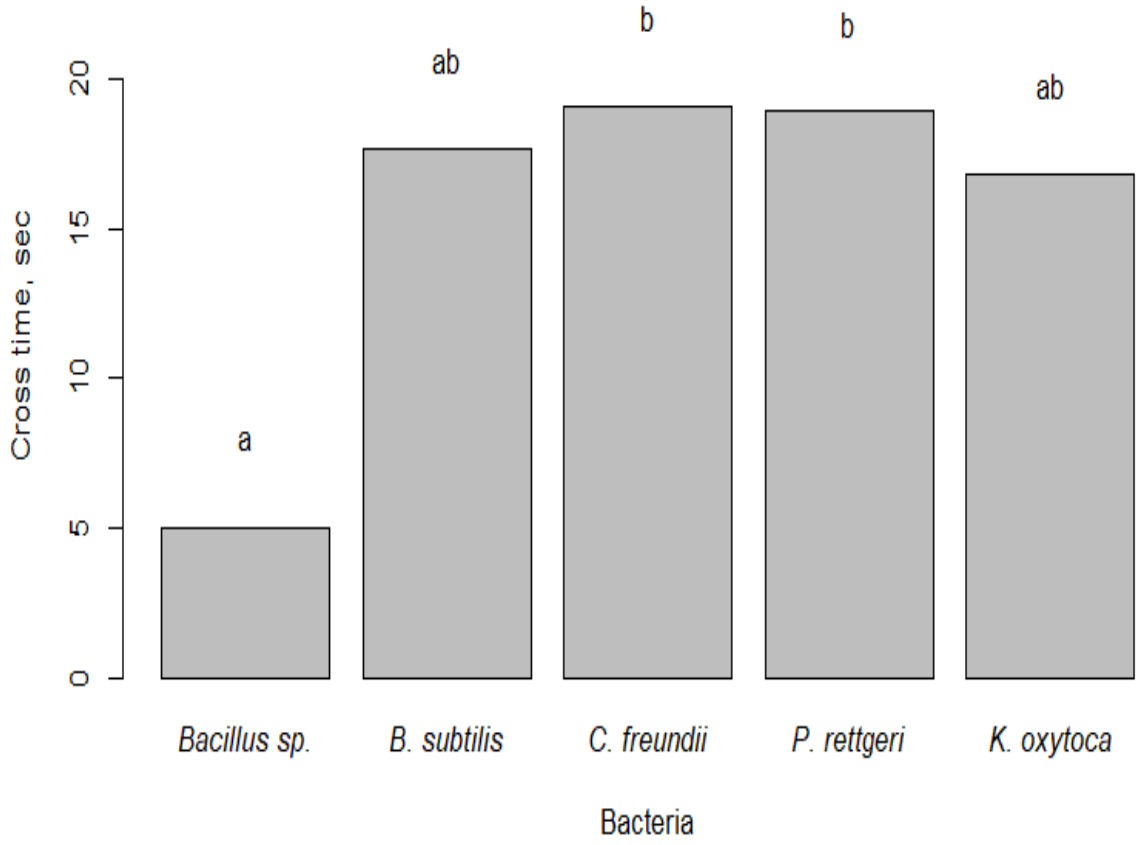

Figure 16: Male mean rank in cross time in wind tunnel experiment. Same letters indicate no significant differences between groups.

Wind tunnel assays showed similar results to the Y-tube olfactometer assays. All females were observed to show attraction towards the various isolates during varying lengths of time. The quickest time for the female Island flies to go past the one metre mark in the wind tunnel towards the odour source was shown for *Bacillus sp.* at 70.3 seconds after release. *C. freundii* showed the slowest reaction times for male Island Flies on average at 107.3 seconds. The odour plume for *C. freundii* had strong sulphur notes and was not probably liked by the flies though they were attracted by the visual cue of the yellow ball.

There was no statistically significant difference noted in the reaction of Island Fly males towards all volatiles in the wind tunnel assay as in Figure 16. Although the results were not statistically significant due to low sample sizes used and low replications, on average all males had the shortest response time for *Bacillus sp.* and longest response time for *C. freundii*.

3.5 EAG Assays

Island Fly antennae were exposed to volatiles by different bacterial isolates contained on pleated filter paper with puffs of the volatiles blown over the antennae attached to electrical electrodes. Any response of the fly antennae to the isolate volatiles was recorded as an electrical impulse (mV) on a computer, as described in Section 2.8.

The EAG responses in mV of Island Fly antennae to the five bacterial isolates and broth is shown in Table 4.

Bacterial Isolate	*Bacillus sp.*	*C. freundii*	*B. subtilis*	*K. oxytoca*	*P. rettgeri*	Broth
Female	0.76	1.13	0.59	0.8	0.93	0.17
Male	0.35	0.33	0.32	0.21	0.13	0.21

Table 4: Responses of Island Fly antennae to Bacterial Volatiles in EAG

During the EAG assays all Island Fly antennae showed responses when exposed to bacterial isolates and nutrient broth. Male and female Island Flies showed different responses to each bacterial isolate (Table 4 and Figure 17). All females generally exhibited higher levels of responses to bacterial isolates than males. In the case of the broth alone, males showed slightly higher antennal responses than females. The highest response was produced by female antennae exposed to C. freundii of 1.13mV while the lowest was of male antennae exposed to P. rettgeri at 0.13mV.

A stronger response was noted when the *C. freundii* volatiles were passed over the antenna. This could be linked to the detection of sulphur (methyl ethyl disulphide and dimethyl tetrasulphide) and other unique compounds noted in this isolate. Results from the Y-tube olfactometer assays showed that Island Fly did not prefer the volatiles from *C. freundii* and were deterred from it, however Robacker *et. al.* (1997) reported attraction of *C. freundii* volatiles to Mexican fruit fly [57, 91]. The largest responses in the EAG assay caused by *C. freundii* corresponds to the Y-tube olfactometer assay. It suggests that the flies have greater resistance to unfavourable odours than the favourable attractive odour of *Bacillus sp.* and probably faster detection capability in nature to avoid sites of unfavourable odours from *C. freundii*.

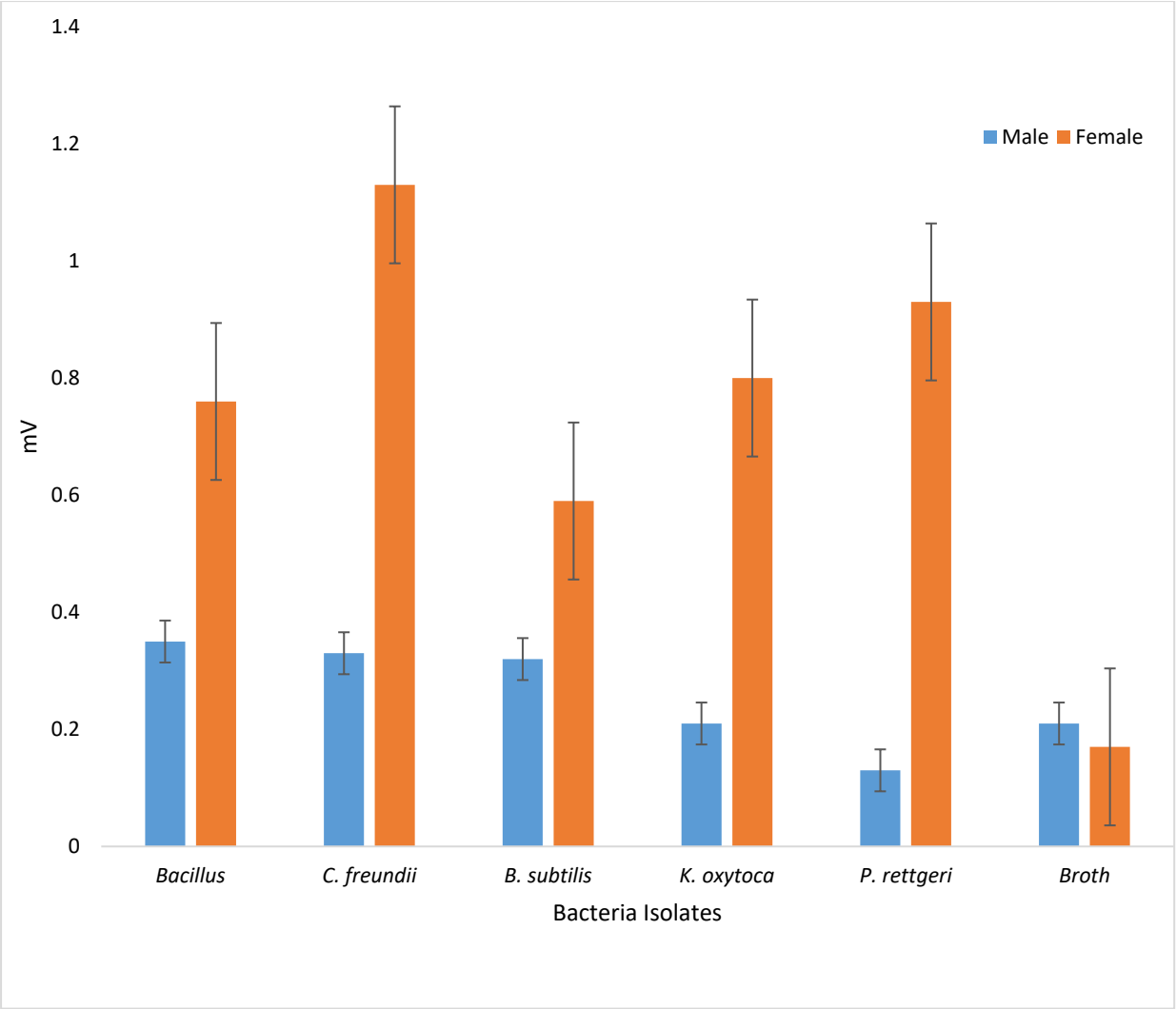

Figure 17: Island Fly male and female EAG responses to bacterial isolates

In the wild, females Island Flies are highly likely to be actively hunting for favourable odours emanating from over-ripe and rotting fruits. The rotting is caused primarily by the bacteria that also produce volatiles which serve as a cue for the female flies to locate and oviposit their eggs. Males may be using the bacterial odours from females as a cue for seeking mates and not primarily as a cue for oviposition places. As reported by Gujjar *et. al.* [79], *B. dorsalis* and *B. cucurbitae* male and female fruit flies have a slightly different cohort of gut bacteria and probably are attracted differently to bacteria species in nature according to their physiological needs, as shown by this EAG assay of males responding lower than females on average.

3.6 GC-MS Analysis

There were some compounds that had long-chain alkanes, CnH2n+2 where n > ~18 that were detected by GC-MS but could not be uniquely identified within the available NIST 21 and NIST 107 library databases. All other compounds described below were only tentatively identified as they were not compared with standard samples, but they showed a high similarity index to the library database MS.

3.6.1 Compound distribution based on peak areas

Volatile compounds produced from the bacterial isolates were identified by GC-MS and were characterised by primary functional groups. The integrated areas for all peaks of the compounds in a class were summed, then ratioed to the total integrated peak area for all compounds from that culture. This process was conducted for all the bacterial isolates (Appendix 2).

The integrated peak areas used in this report have to be treated with caution as no standardisation or normalisation was undertaken in the assays. Variations in integrated area may be due to variations in sampling procedures or variations in bacterial culture behaviour (biological activities) and have not been accounted for, thus the relative areas below are tentative. The nutrient broth had a higher relative amount of aldehydes based on the integrated peak areas in comparison to the other isolates and was very similar with *C. freundii* and *K. oxytoca* proportions (Figure 18). However, *C. freundii* had a higher relative amount of acids in comparison to other isolates and broth, and no esters were produced by it. The relative amount of ketones was the highest in the broth and only in lower relative amounts in *P. rettgeri*. Ketones were not detected in all other bacteria isolates. *P. rettgeri* had the highest relative amounts of alcohols. Sulphurous compounds were seen in higher relative quantities in *Bacillus sp*. and *B. subtilis* than other isolates. Alcohols and esters were in low abundance in *Bacillus sp*.

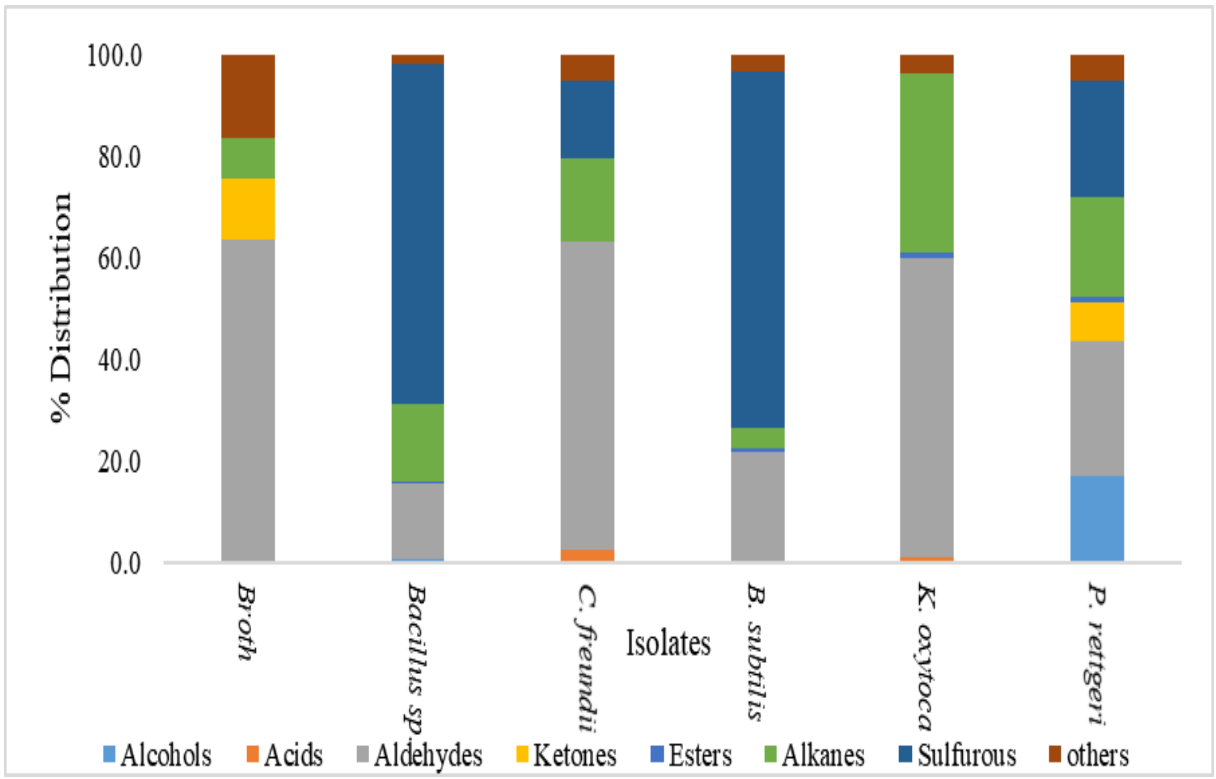

Figure 18: Compound class proportions based on integrated peak areas

3.6.2 Compound distribution based on number of types in the bacterial isolates

Bacillus sp. had many different types of alcohols and esters in comparison to other isolates after the incubation period. Alkane types had relative increases in *Bacillus sp.* from nutrient broth levels. Ketones were detected in the nutrient broth but were not detected in all the bacterial isolates except in relatively lower quantities in *P. rettgeri* (Figure 19). As reported by Onaca et. al *Pseudomonas veronii* bacteria was isolated and its genes were cloned in *Escherichia coli*. Then a DNA sequence analysis of a 15-kb fragment was conducted, which revealed three genes involved in methyl ketone degradation [80]. *C freundii* and *K. oxytoca* had many types of acids detected in them.

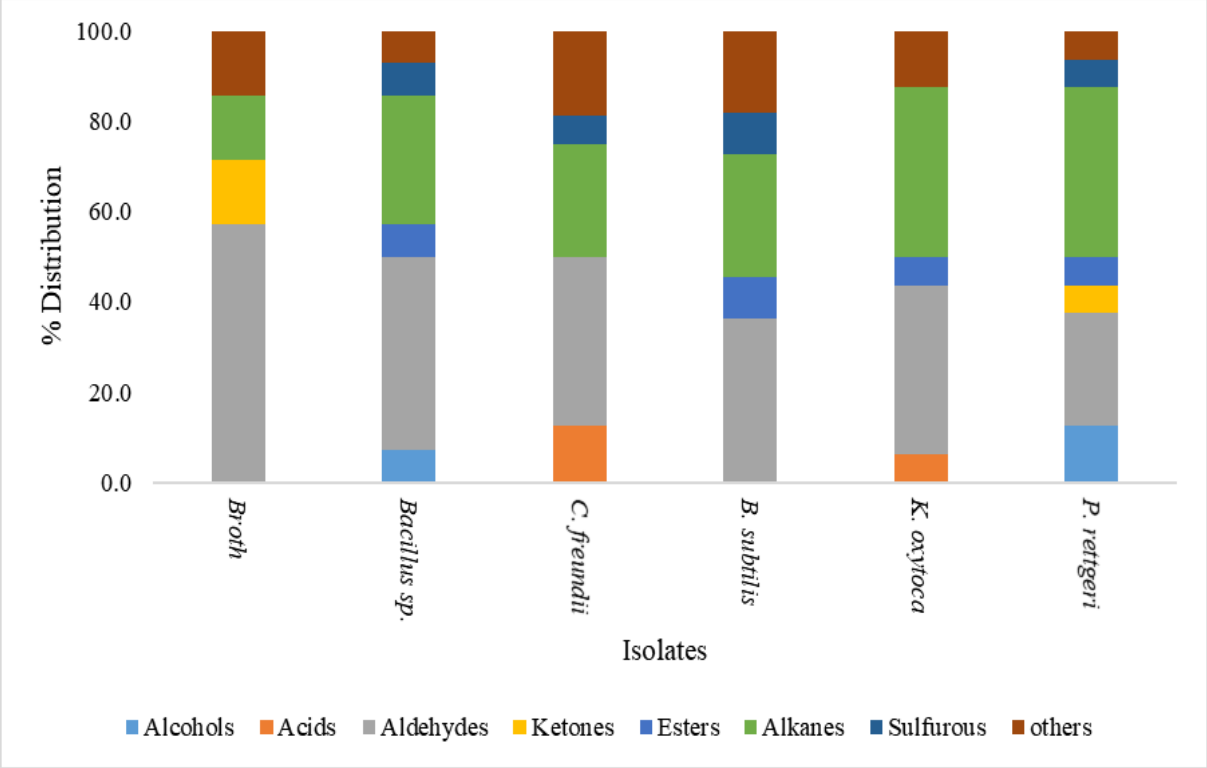

Figure 19: Compound distribution based on amount in the bacterial isolates

3.6.3 Common compounds

There were a number of compounds common to all of the incubated bacterial isolates and the broth itself. However, the amount of these compounds in the headspace had either increased or decreased in comparison to the initial levels in the nutrient broth. This suggests that the bacterial isolates were either utilising these compounds from the nutrient broth or producing them.

The following were common compounds found in all bacterial isolates and the broth:

2-ethylhexanal, octanal, 2,8-dimethylundecane, undecane, nonanal, 2-ethylhexyl acetate, dodecane, decanal, undecanal, dodecanal, hexadecanenitrile, [1,1':3',1"-terphenyl]-2'-ol.

Octanal was found in all the bacterial isolates. The value of the summed integrated peak area showing greatest relative increase in comparison to broth levels was noted in *C. freundii* isolates (266 x) and the least was in *Bacillus sp.* (120 x). Similar relative increases were noted for *B. subtilis* (171 x), followed by *P. rettgeri* (194 x) and *K. oxytoca* (228 x).

A similar effect was noted for decanal. The value of the summed integrated peak area showing greatest relative increase was for *C. freundii* (411 x) and the least by *Bacillus sp.* (148 x). Similar relative increases were noted for *B. subtilis* (179 x), *P. rettgeri* (193 x) and *K. oxytoca* (256 x).

The above results suggest that *C. freundii* was producing most of these compounds more profusely during the 24 hours incubation period in comparison to *Bacillus sp.* An odour that is composed of fewer types of compounds may be linked to the odour preferred by the flies for attraction as noted for *Bacillus sp*.

3.6.4 Compounds common to all bacterial isolates excluding broth

The GC-MS results showed that the bacterial isolates of Island Fly produced common compounds not detected in the nutrient broth head space. The absence of these compounds from the nutrient broth head space (control) suggests that the bacterial isolates had produced these compounds during the 24 hours incubation process.

The following were compounds found in all bacterial isolates excluding the broth:

hexanal, 2,4-dimethylheptane, 2,4-dimethyl-1-heptene, 4-methyloctane, 2-ethyl-1-hexanol, 3,6-dimethylundecane, nonanoic acid, phthalic anhydride, *n*-decanoic acid, tetradecane, methyl eugenol, geranyl acetone (6,10-dimethyl-5,9-undecadien-2-one), *n*-hexadecanoic acid.

The odour of many known artefacts, such as the phthalate anhydride and adipate plasticisers, bis(2-ethylhexyl) phthalate and its degradation product 2-ethylhexanol are produced by bacteria [53] (Schulz, 2007). Methyl eugenol is a well-known compound associated with attracting fruit flies of several species (*B. dorsalis, B. zonata* and *B. carambolae*) and used extensively in lure traps for control and management [81]. Island Fly is attracted to Cue lure and not methyl eugenol lure. The results show that methyl eugenol is being produced by Island Fly gut bacteria in combination with other odours, thus the odour of methyl eugenol may be masked in the mixture and not stand alone as a distinct odour, thus not being attractive. Geranyl acetone is another compound found in all Island Fly bacterial isolates that has an odour associated with fresh rose, leaf floral, green magnolia and

aldehydic fruity blends notes. These odours may be cues in nature for fruit flies in their natural habitats being attracted to vegetation and fruits [82].

Nonanoic acid was produced by all bacterial isolates. The greatest increase was noted in *C. freundii* isolates (100 x), followed by *Bacillus sp*. (77 x), *K. oxytoca* (66 x) and *B. subtilis* (36 x). Nonanoic acid was the least abundant in *P. rettgeri* (34 x).

3.6.5 Compounds unique to a particular bacterial isolate

The bacterial isolates had some compounds that were only produced by a particular bacterial isolate and not by the other isolates, making them unique compounds for that particular isolate. The response of the Island Fly to a particular isolate could be linked to one compound or a combination of these unique compounds.

The *Bacillus sp.* isolate was noted to emit the least number (ten) of unique compounds and showed the greatest levels of attraction in the Y-tube and wind tunnel assays. The most compounds (25) were emitted by *P. rettgeri*, but the Island Flies were moderately attracted to the isolate. However, *C. freundii* was noted to deter the Island Flies and had moderate numbers of compounds (16), which were mainly acids.

The following compounds were unique to particular isolates:

Bacillus sp.: 2,2,3,4,6,6-hexamethylheptane, 6-ethyl-2-methyloctane, cyclododecane, 1-tridecanol, 1H-isoindole-1,3(2H)-dione, 2-ethyl-1-dodecanol, 1-octadecene, pentadecanal, 1-chlorooctadecane, heptadecanenitrile

Citrobacter freundii: methanethiol, methyl ethyl disulphide, 2,4,6-trimethylheptane, 2,4-dithiapentane, phenylacetaldehyde, S-methyl methanethiosulfonate, 1-phenyl-1,2-propanedione, benzoylformic acid, dimethyl tetrasulphide, undecanal, dodecanal, 2-cyanobenzoic acid, hexadecanol, 1-tridecene

Bacillus subtilis: α-pinene, benzaldehyde, undecane, 3,6-dimethylundecane, 1-dodecene, 5-butylbonane, (*E*)-9-eicosene, 4-(4-hydroxyphenyl)-2-butanone, octadecanal, 1-octadecene, methyl hexadecanoate (methyl palmitate), eicosane

Klebsiella oxytoca: 3-methylbutanoic acid, 4,8-dimethylundecane, 4,6-dimethyldodecane, 4,7-dimethylundecane, 4-ethyl-2,2,6,6-tetramethylheptane, 3,4-dimethyldecane, 2-butyl-1-octanol, methyl 7,9-tridecadienyl ether, tridecanal, dioctyl ether, tetradecanal, 1-octadecene, octadecanal, 1-heneicosyl formate, isopropyl palmitate, tetratetracontane

Providencia rettgeri: *N,N*-dimethyl-3-buten-1-amine, *p*-xylene, 3-heptanone, 1,4-dichlorobenzene, 2,2,3-trimethylnonane, 2,8,8-trimethyldecane, 2,4-dimethylundecane, dimethyl tetrasulphide, 4-methyldodecane, decanenitrile, tetradecane, 1-nonadecanol, 4,6-dimethyldodecane, 1-tridecene, (*E*)-3-octadecene, tritetracontane, heneicosane, hexadecane, diethyl phthalate, 1-chloro-hexadecane, 1-tetradecanol, *N*-butylbenzenesulfonamide, diphenylpropanetrione, hexadecanal, (*E,E*)-7,11,15-trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene

Methanethiol has high volatility, and has also been reported in a few of these bacterial species such as *Pseudomonas sp., Serratia sp. and Enterobacter sp.* [83].

2,4-Dithiapentane is an organosulfur compound known to be one component of truffle flavour [84]. It is used as a primary aromatic additive in truffle oil and is a colourless liquid with a strong odour.

Phenylacetaldehyde is an insect attractant and is used in black light traps for some insect pests [85-87]. It is a component of floral scent and is an intermediate in a variety of biochemical pathways.

3.6.6 Compounds present in either *Bacillus sp.* or *Citrobacter freundii*

The Island Flies, when exposed to the odours of the bacterial isolates *Bacillus sp.* and *C. freundii*, showed contrasting reactions; attraction to *Bacillus sp.* and deterrence from *C. freundii*. A similar effect was noted in the previous study in Adelaide by Dr Crisp's laboratory group [13]. These contrasting behaviours may be linked to the different compounds emitted by the two species of bacteria or by similar compounds produced by these species but in different proportions. It was noted that *Bacillus sp.* produced fewer different compounds (21) than *C. freundii* (32). The differences between the productions of unique compounds by these two bacterial isolates may account for the behavioural reactions of Island Flies towards the cultures' emissions.

Bacillus sp.: 2,2,3,4,6,6-hexamethylheptane, 5-ethyl-2-methyloctane, 2,6,6-trimethyloctane, 6-ethyl-2-methyloctane, 1-chlorotetradecane, cyclododecane, 1-dodecene, 4,6-dimethylundecane, 4,8-dimethylundecane, 4,6-dimethyldodecane, 1-tridecanol, 1H-isoindole-1,3(2H)-dione, 2-ethyl-1-dodecanol, tetradecanal, 1-octadecene, pentadecanenitrile, pentadecanal, 1-chlorooctadecane, heptadecanenitrile, heneicosane.

Citrobacter freundii: methanethiol, methyl ethyl disulphide, 2,4,6-trimethylheptane, 2,4-dithiapentane, benzeneacetaldehyde, S-methyl methanethiosulphonate, 1-phenyl-1,2-propanedione, benzoylformic acid, 1-chlorododecane, benzoic acid, octanoic acid, 1-tetradecene, 2,6-dimethylundecane, dimethyl

tetrasulphide, undecanal, tridecane, dodecanal, 1-tetradecene, *o*-cyanobenzoic acid, hexadecanol, 1-tridecene, dodecanoic acid, hexadecane, tetradecanal, hexadecane, tetradecanoic acid, 1-heptadecene, pentadecanoic acid, octadecanal, octadecanenitrile, octadecanoic acid, docosane.

The differences between the volatiles produced by these two bacterial isolates are that *Bacillus sp.* is dominated by alkanes, some alcohols and aldehydes and has nitrogen containing compounds (1H-isoindole-1,3(2H)-dione, pentadecanenitrile, heptadecanenitrile). *C. freundii* produced lots of sulphur-containing compounds (methanethiol, methyl ethyl disulphide, 2,4-dithiapentane, S-methyl methanethiosulphonate, dimethyl tetrasulphide) and lots of acids (benzoylformic acid, benzoic acid, octanoic acid, *o*-cyanobenzoic acid, dodecanoic acid, tetradecanoic acid, pentadecanoic acid, octadecanoic acid). It also had alcohols, aldehydes and nitrogen compounds (*o*-cyanobenzoic acid, octadecanenitrile).

3.6.7 Future investigations for compound identification

Future investigations will look at confirmation of the volatile compounds through comparison with authentic samples obtained commercially or *via* synthesis. These authentic samples will also allow response studies with Island Fly, to see their individual effects as well as those of differing mixtures of volatiles.

CHAPTER 4: CONCLUSIONS & FUTURE DIRECTIONS

4.1 Conclusions

A F1 generation of Island Fly was successfully reared in the laboratory as described in Chapter 2, using modified rearing procedures from that used at SARDI, Adelaide [13, 63]. The Island Fly cultures were noted in the experiments to adapt to the controlled environmental rooms, improved larval gel diets, diet combined oviposition containers and to addition of wheat germ oil. The performance of the colony may be improved for laboratory rearing by overcoming the inherent seasonal pattern shown by Island Flies.

Bacterial isolates were obtained from the gut of female Island Flies and incubated to obtain colonies. Most isolates were identified as soil dwelling bacteria found on plants as well. Generally, on average the female Island Flies showed strong attraction for *Bacillus sp.* isolates while males were moderately attracted. *C. freundii* was noted to be non-attractive and both male and female flies were deterred by its odours. There were no statistical significant differences seen in the responses of Island Flies when exposed to the volatile emissions of these various isolates in all the assays. Females on average showed quicker responses towards volatiles from *Bacillus sp.* and not much preference for the other isolates. Females were noted to avoid and be deterred by *C. freundii* in the Y-tube olfactometer bioassays. All flies showed varying levels of responses in the EAG assays and females generally exhibited higher levels of responses to bacterial isolates than males. In the case of the broth alone, males showed slightly higher antennal responses than females in EAG assays.

The preferences towards or away from the isolates may be linked to the unique volatile organic compounds that were being produced by these bacterial isolates. *Bacillus sp.* was dominated by alkanes, alcohols and aldehydes and had nitrogen containing compounds (1H-isoindole-1,3(2H)-dione, pentadecanenitrile, heptadecanenitrile). Island Flies deterrence to *C. freundii* may be due to compounds containing acids (benzoylformic acid, benzoic acid, dodecanoic acid, tetradecanoic acid, pentadecanoic acid and octadecanoic acid) and sulphur containing compounds (methanethiol, methyl ethyl disulphide, 2,4-dithiapentane, S-methyl methanethiosulphonate, dimethyl tetrasulphide). Many other compounds were noted to be produced uniquely by the bacterial isolates and could hold some clues on the attraction of Island Flies towards or deterred away from them. However, due to time constraints further analysis of these singular compounds was not possible in this project.

4.2 Future Directions

The Y-tube olfactometer assays in this project showed Island Flies to have an attraction and rapid reaction towards *Bacillus sp.*, and deterrence towards *C. freundii*. These results have shown that all isolates shall be further investigated with more population samples and replications in the laboratory

for confirmations. The components of the bacterial isolates shall be chemically identified through comparison with authentic samples in terms of retention time and MS fragmentation similarity to the MS library databases. Pure compounds and their blends may be procured or synthesised. These compounds can be used for conducting laboratory and field trials and for confirmation of attractions and deterrence with greater confidence for Island Flies.

This project was conducted over a period of nine months only and the time frame was not sufficient to rear and domesticate a colony of Island Fly from the wild into laboratory colony in a short period. For many assays to be conducted and replicated multiple times, a large population of Island fly in laboratory was needed. Initial establishment of the Island Flies colony with large populations (>5000 flies) is needed from the wild. These flies shall be reared over several generations (at least greater than four generations) to assist in having a genetically robust colony. This robust colony shall continually produce flies in large quantities to allow for more replications in the various assays and get higher confidence in the results.

Another important aspect is the conduct of laboratory testing of flies is often too artificial to completely mimic natural behaviour of the flies. Thus, conduct of assays in cage trials and expanding into field trials would be of value. A large population of Island Flies is initially required to carry out such trials and be replicated for better accuracy of the results.

The recent findings of Gujjar *et. al.* [79] demonstrated that male fruit flies have a different cohort of bacteria within their biome than females. This project should therefore also be conducted with the bacterial isolates obtained from male Island Flies to investigate any correlations. Bacterial isolate experimentation from male Island Flies was not possible in this project due to limited time for completion and submission of thesis.

Male Island Flies have been observed to provide a nuptial gift to females. The gift is a regurgitate that the male lays neatly on a substrate surface and then hovers around the demarcated area. The area is defended aggressively by the male that provided the regurgitate and only females are permitted to feed on the regurgitate. While the female is feeding, the male approaches the female for copulation. It is understood that the regurgitate may contains some volatiles within it, whereby the females picks up those odours, gets attracted and is able to make her way to the actual demarcated area and meets the male for copulation. Future chemical analysis of this regurgitate and biological assays to investigate its effects could be fruitful.

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APPENDIX 1:

SI No.	Ingredient	Quantity	Units	Source
1	Dried Yeast	183.6	g	Aussie Health Products
2	White Sugar	109.2	g	Coles Supermarket
3	Methyl Parabens	1.8	g	Sigma Aldrich
4	Citric Acid	21	g	Sigma Aldrich
5	Wheat Germ Oil (Organic)	1.8	ml	Melrose Health Group
6	Sodium Benzoate	1.8	g	Sigma Aldrich
7	Bacteriological Agar	9	g	Sigma Aldrich
8	Water	900	ml	Boiling water

Table 5: Modified Chang's Gel Diet[66] for Island Fly

APPENDIX 2

Table 6: All compounds emitted by the Bacterial Isolates of Island fly

Retention Time	**Broth**	***Bacillus sp.***	***Citrobacter freundii***	***Bacillus subtilis***	***Klebsiella oxytoca***	***Providencia rettgeri***
2.32	-	-	Methanethiol	-	-	-
2.47	-	-	-	-	-	3-Buten-1-amine, N,N-dimethyl-
2.59	-	-	-	Carbon disulfide	Carbon disulfide	Carbon disulfide

2.67	-	-	-	-	Butanoic acid, 3-methyl-	-
2.79	-	-	-	Acetic acid	-	Acetic acid
3.99	Disulfide, dimethyl	Disulfide, dimethyl	-	Disulfide, dimethyl	Disulfide, dimethyl	Disulfide, dimethyl
4.26	-	Toluene	-	Toluene	Toluene	-
4.53	-	-	-	-	-	Butanoic acid
4.70	-	Hexanal	Hexanal	Hexanal	Hexanal	Hexanal
5.08	-	Heptane, 2,4-dimethyl-	Heptane, 2,4-dimethyl-	Heptane, 2,4-dimethyl-	Heptane, 2,4-dimethyl-	Heptane, 2,4-dimethyl-
5.33	-	-	Methyl ethyl disulphide	-	-	-
5.39	-	2,4-Dimethyl-1-heptene	2,4-Dimethyl-1-heptene	2,4-Dimethyl-1-heptene	2,4-Dimethyl-1-heptene	2,4-Dimethyl-1-heptene
5.73	-	Octane, 4-methyl-	Octane, 4-methyl-	Octane, 4-methyl-	Octane, 4-methyl-	Octane, 4-methyl-
5.84	-	Benzene, 1,2-dimethyl-	-	Benzene, 1,2-dimethyl-	Benzene, 1,2-dimethyl-	-
5.83	-	-	-	-	-	p-Xylene
5.91	-	-	Heptane, 2,4,6-trimethyl-	-	-	-

6.17	-	-	2,4-Dithiapentane	-	-	-
6.06	-	-	-	-	-	3-Heptanone
6.34	-	Heptanal	Heptanal	Heptanal	-	Heptanal
7.03	-	-	-	alpha-Pinene	-	-
7.35	Hexanal, 2-ethyl-	Hexanal, 2-ethyl-	Hexanal, 2-ethyl-	Hexanal, 2-ethyl-	Hexanal, 2-ethyl-	Hexanal, 2-ethyl-
7.46	-	Benzaldehyde	Benzaldehyde	-	-	-
7.68	-	Dimethyl trisulfide	Dimethyl trisulfide	-	Dimethyl trisulfide	Dimethyl trisulfide
7.74	-	Decane, 2,2,3-trimethyl-	-	Decane, 2,2,3-trimethyl-	-	-
7.51	-	-	-	Benzaldehyde	-	-
7.80	-	-	-	Undecane	-	-
7.90	-	Phenol	-	Phenol	-	Phenol
7.93	-	alpha-Methylstyrene	alpha-Methylstyrene	alpha-Methylstyrene	alpha-Methylstyrene	-
8.11	-	Octane, 2,3,3-trimethyl-	-	1-Decene	-	Octane, 2,3,3-trimethyl-
8.21	Cyclotetrasiloxane, octamethyl-	Cyclotetrasiloxane, octamethyl-	Cyclotetrasiloxane, octamethyl-	Cyclotetrasiloxane, octamethyl-	Cyclotetrasiloxane, octamethyl-	Cyclotetrasiloxane, octamethyl-

8.34	Octanal	Octanal	Octanal	Octanal	Octanal	Octanal
8.44	-	Benzene, 1,3-dichloro-	Benzene, 1,4-dichloro-	Benzene, 1,3-dichloro-	Benzene, 1,2-dichloro-	-
8.50	-	Octane, 3,3-dimethyl-	Heptane, 3,3,5-trimethyl-	Octane, 3,3-dimethyl-	-	Octane, 3,3-dimethyl-
8.54	-	Nonane, 2,2,3-trimethyl-	Nonane, 2,2,3-trimethyl-	Nonane, 2,2,3-trimethyl-	Nonane, 2,2,3-trimethyl-	-
8.56	-	-	-	-	Undecane, 4,8-dimethyl-	-
8.55	-	-	-	-	Dodecane, 4,6-dimethyl-	-
8.46	-	-	-	-	-	Benzene, 1,4-dichloro-
8.56	-	-	-	-	-	Nonane, 2,2,3-trimethyl-
8.59	-	Nonane, 4,5-dimethyl-	Nonane, 4,5-dimethyl-	Nonane, 4,5-dimethyl-	-	Nonane, 4,5-dimethyl-
8.78	-	Undecane, 3,6-dimethyl-	-	-	Undecane, 3,6-dimethyl-	-
8.79	-	-	-	-	Heptane, 4-ethyl-2,2,6,6-tetramethyl-	Heptane, 4-ethyl-2,2,6,6-tetramethyl-
8.82	-	-	Nonane, 3-methyl-	Nonane, 3-methyl-	Decane, 2,2-dimethyl-	Decane, 2,2-dimethyl-

8.83	-	Heptane, 2,2,3,4,6,6-hexamethyl-	-	-	-	-
8.92	-	1-Hexanol, 2-ethyl-	1-Hexanol, 2-ethyl-	1-Hexanol, 2-ethyl-	1-Hexanol, 2-ethyl-	1-Hexanol, 2-ethyl-
8.98	-	D-Limonene	-	D-Limonene	-	-
9.03	-	-	-	-	Undecane, 4,7-dimethyl-	-
9.06	-	Octane, 3-ethyl-2,7-dimethyl-	-	-	Octane, 3-ethyl-2,7-dimethyl-	Octane, 3-ethyl-2,7-dimethyl-
9.08	-	-	-	Decane, 3,7-dimethyl-	Decane, 3,7-dimethyl-	Decane, 3,7-dimethyl-
9.09	-	Nonane, 3,7-dimethyl-	Decane, 3,7-dimethyl-	Octane, 3-ethyl-2,7-dimethyl-	-	-
9.16	-	Undecane, 3,6-dimethyl-	Undecane, 3,6-dimethyl-	Undecane, 2,5-dimethyl-	Undecane, 3,6-dimethyl-	Octane, 4-ethyl-
9.19	-	-	-	-	-	Undecane, 3,6-dimethyl-
9.44	-	-	-	-	Octane, 2,3,6,7-tetramethyl-	Octane, 2,3,6,7-tetramethyl-
9.21	-	-	Benzeneacetaldehyde	-	-	-
9.49	-	-	-	-	Heptane, 4-ethyl-2,2,6,6-tetramethyl-	-

9.47	-	Octane, 2,3,6,7-tetramethyl-	Octane, 2,3,6,7-tetramethyl-	Nonane, 5-(2-methylpropyl)-	Heptane, 5-ethyl-2,2,3-trimethyl-	Heptane, 5-ethyl-2,2,3-trimethyl-
9.54	-	-	-	-	Nonane, 3-methyl-	Nonane, 3-methyl-
9.55	-	Heptane, 5-ethyl-2,2,3-trimethyl-	Nonane, 3-methyl-	Heptane, 5-ethyl-2,2,3-trimethyl-	Undecane, 4,7-dimethyl-	Decane, 3,6-dimethyl-
9.58	-	-	Undecane, 5-methyl-	-	Undecane, 5-methyl-	-
9.59	-	-	-	-	Decane, 3,4-dimethyl-	-
9.61	-	Octane, 5-ethyl-2-methyl-	-	Octane, 5-ethyl-2-methyl-	-	-
9.64	-	-	S-Methyl methanethiosulphonate	-	-	-
9.67	-	-	-	-	Dodecane, 3-methyl-	Dodecane
9.70	-	Undecane, 5,7-dimethyl-	Heptane, 4-ethyl-2,2,6,6-tetramethyl-	-	Heptane, 2,2,3,4,6,6-hexamethyl-	-
9.75	-	Dodecane, 3-methyl-	-	Octane, 5-ethyl-2-methyl-	-	Heptane, 2,2,3,4,6,6-hexamethyl-
9.84	-	Acetophenone	-	Acetophenone	-	Acetophenone

9.87	-	-	1,2-Propanedione, 1-phenyl-	-	-	-
9.89	-	-	-	Undecane, 3,6-dimethyl-	-	-
9.94	-	-	Benzoylformic acid	-	-	-
10.04	-	-	-	-	-	Decane, 2,8,8-trimethyl-
10.05	Undecane, 2,8-dimethyl-	Undecane, 2,8-dimethyl-	Undecane, 2,8-dimethyl-	Undecane, 2,8-dimethyl-	Undecane, 2,8-dimethyl-	Undecane, 2,8-dimethyl-
10.06	-	Octane, 2,6,6-trimethyl-	-	Octane, 2,6,6-trimethyl-	-	-
10.43	-	-	-	1-Dodecene	-	-
10.48	-	-	-	Nonane, 5-(2-methylpropyl)-	Nonane, 5-(2-methylpropyl)-	-
10.53	-	-	-	Nonane, 5-butyl-	-	-
10.61	-	Octane, 6-ethyl-2-methyl-	-	-	-	-
10.61	-	-	Decane, 3,7-dimethyl-	Decane, 3,7-dimethyl-	-	-
10.62	Undecane	Undecane	Undecane	Undecane	Undecane	Undecane

10.62	-	-	-	-	Decane, 3,7-dimethyl-	Decane, 3,7-dimethyl-
10.71	Nonanal	Nonanal	Nonanal	Nonanal	Nonanal	Nonanal
10.81	-	Decane, 3,7-dimethyl-	-	Decane, 3,7-dimethyl-	Decane, 3,7-dimethyl-	-
11.10	-	-	-	Octane, 2,3,6,7-tetramethyl-	Decane, 2,2,5-trimethyl-	-
11.23	-	Heptane, 4-ethyl-2,2,6,6-tetramethyl-	Heptane, 4-ethyl-2,2,6,6-tetramethyl-	-	Heptane, 4-ethyl-2,2,6,6-tetramethyl-	-
11.25	-	-	-	Decane, 2,2,6-trimethyl-	-	Decane, 2,2,6-trimethyl-
11.89	Acetic acid, 2-ethylhexyl ester	Acetic acid, 2-ethylhexyl ester	Acetic acid, 2-ethylhexyl ester	Acetic acid, 2-ethylhexyl ester	Acetic acid, 2-ethylhexyl ester	Acetic acid, 2-ethylhexyl ester
12.19	-	-	-	-	Tetradecane, 1-chloro-	-
12.23	-	Tetradecane, 1-chloro-	Dodecane, 1-chloro-	-	-	-
12.23	-	-	-	Decane, 1-chloro-	Decane, 1-chloro-	-
12.27	-	-	-	-	-	Dodecane, 1-chloro-
12.39	-	-	Benzoic Acid	Benzoic Acid	-	Benzoic Acid
12.39	-	Dodecanal	Dodecanal	Dodecanal	-	-

12.51	-	-	Octanoic Acid	Octanoic Acid	Octanoic Acid	Octanoic Acid
12.95	-	-	-	-	Cyclododecane	Cyclododecane
12.97	-	Cyclododecane	-	-	-	-
12.97	-	-	1-Tetradecene	-	1-Tetradecene	1-Tetradecene
12.98	-	1-Dodecene	-	1-Dodecene	1-Dodecene	-
13.19	Dodecane	Dodecane	Dodecane	Dodecane	Dodecane	Dodecane
13.34	Decanal	Decanal	Decanal	Decanal	Decanal	Decanal
13.53	-	-	-	-	-	Undecane, 2,4-dimethyl-
13.57	-	Undecane, 4,6-dimethyl-	-	Undecane, 4,6-dimethyl-	-	-
13.56	-	-	Undecane, 2,6-dimethyl-	Undecane, 2,6-dimethyl-	Undecane, 2,6-dimethyl-	Undecane, 2,6-dimethyl-
13.57	-	-	-	-	1-Octanol, 2-butyl-	-
13.61	-	-	Dimethyl tetrasulphide	-	-	-
13.62	-	-	-	-	-	Undecane, 4,6-dimethyl-
13.70	-	-	-	-	-	Dimethyl tetrasulphide

13.78	-	Undecane, 4,8-dimethyl-	-	Undecane, 4,8-dimethyl-	-	Undecane, 4,8-dimethyl-
14.07	-	-	-	-	Dodecane, 2,6,11-trimethyl-	Dodecane, 4-methyl-
14.49	-	-	-	Dodecane, 4,6-dimethyl-	-	Dodecane, 4,6-dimethyl-
14.75	-	-	-	-	-	Dodecane, 4-methyl-
14.86	-	Undecane, 2,4-dimethyl-	-	Undecane, 2,4-dimethyl-	Nonanoic acid	Undecane, 2,4-dimethyl-
14.95	-	Nonanoic acid	Nonanoic acid	Nonanoic acid	-	Nonanoic acid
14.92	-	-	Undecanal	-	-	-
15.22	-	Dodecane, 4,6-dimethyl-	-	Dodecane, 4,6-dimethyl-	Dodecane, 4,6-dimethyl-	Dodecane, 4,6-dimethyl-
15.36	-	-	-	-	-	Decanenitrile
15.44	-	-	-	-	-	Tetradecane
15.49	-	-	-	1-Tridecene	-	1-Tridecene
15.63	-	-	Tridecane	Tridecane	Tridecane	Tridecane
15.73	-	-	-	-	-	Dodecane, 4,6-dimethyl-
15.79	Undecanal	Undecanal	Undecanal	Undecanal	Undecanal	Undecanal
15.83	-	-	-	-	-	1-Nonadecanol
15.97	-	Phthalic anhydride	Phthalic anhydride	Phthalic anhydride	Phthalic anhydride	Phthalic anhydride

16.21	-	-	-	-	-	Dodecane, 4,6-dimethyl-
16.97	-	n-Decanoic acid	n-Decanoic acid	n-Decanoic acid	n-Decanoic acid	n-Decanoic acid
17.07	-	-	Dodecanal	-	-	-
17.45	-	-	-	-	-	1-Tridecene
17.44	-	-	-	-	-	3-Octadecene, (E)-
17.42	-	-	1-Tetradecene	1-Tetradecene	1-Tetradecene	-
17.47	-	1-Tridecanol	-	-	-	-
17.58	-	Tetradecane	Tetradecane	Tetradecane	Tetradecane	Tetradecane
17.68	-	Benzene, 1,2-dimethoxy-4-(2-propenyl)-	Benzene, 1,2-dimethoxy-4-(2-propenyl)-	Benzene, 1,2-dimethoxy-4-(2-propenyl)-	Benzene, 1,2-dimethoxy-4-(2-propenyl)-	Benzene, 1,2-dimethoxy-4-(2-propenyl)-
17.75	Dodecanal	Dodecanal	Dodecanal	Dodecanal	Dodecanal	Dodecanal
18.00	-	Heptadecane	-	-	-	Heptadecane
18.50	-	5,9-Undecadien-2-one, 6,10-dimethyl-	5,9-Undecadien-2-one, 6,10-dimethyl-, (E)-	5,9-Undecadien-2-one, 6,10-dimethyl-	5,9-Undecadien-2-one, 6,10-dimethyl-	5,9-Undecadien-2-one, 6,10-dimethyl-, (E)-
18.66	-	-	o-Cyanobenzoic acid	-	-	-
18.67	-	1H-Isoindole-	-	-	-	-

		1,3(2H)-dione				
19.05	-	-	Hexadecanol	-	-	-
19.06	-	-	-	-	Methyl 7,9-tridecadienyl ether	-
19.09	-	1-Pentadecene	1-Pentadecene	-	1-Pentadecene	1-Pentadecene
19.09	-	-	-	-	-	Tritetracontane
19.11	-	2-Ethyl-1-dodecanol	-	-	-	-
19.11	-	-	1-Tridecene	-	-	-
19.11	-	-	-	9-Eicosene, (E)-	-	-
19.16	-	-	-	-	Heptadecane	Heptadecane
19.20	-	-	-	-	-	Heneicosane
19.27	-	2-Butanone, 4-(4-methoxyphenyl)-	2-Butanone, 4-(4-methoxyphenyl)-	2-Butanone, 4-(4-methoxyphenyl)-	-	-
19.36	-	-	-	-	Tridecanal	-
19.41	-	Tetradecanal	-	Tetradecanal	-	Tetradecanal
19.80	-	Phenylmaleic anhydride	Phenylmaleic anhydride	Phenylmaleic anhydride	-	Phenylmaleic anhydride
19.84	-	-	-	-	-	Hexadecane
20.06	-	-	-	2-Butanone, 4-(4-hydroxyphenyl)-	-	-

20.13	-	-	Dodecanoic acid	-	-	-
20.52	-	-	1-Pentadecene	-	1-Pentadecene	-
20.55	-	-	1-Pentadecanol	1-Heptadecene	1-Heptadecene	1-Octadecene
20.57	-	1-Octadecene	-	-	-	-
20.63	-	-	Hexadecane	Hexadecane	Hexadecane	-
20.75	-	-	Tetradecanal	Tetradecanal	Tetradecanal	1-Heptadecene
20.67	-	-	-	-	-	Diethyl Phthalate
20.84	-	Tetradecanal	-	-	-	Tetradecanal
21.46	-	-	-	-	Octane, 1,1'-oxybis-	-
21.66	-	-	-	-	-	Hexadecane, 1-chloro-
21.85	-	1-Pentadecene	1-Heptadecene	-	-	-
21.86	-	-	-	4-Trifluoroacetoxytridecane	-	4-Trifluoroacetoxytridecane
21.87	-	-	-	-	-	1-Tetradecanol
21.89	-	-	-	-	Tetradecanenitrile	Tetradecanenitrile
21.93	-	-	Hexadecane	Hexadecane	-	-
22.11	-	-	-	-	Tetradecanal	-
22.14	-	-	Hexadecanal	-	-	Hexadecanal
22.15	-	-	-	Octadecanal	-	-

22.69	-	-	Tetradecanoic acid	-	Tetradecanoic acid	Tetradecanoic acid
23.03	-	-	-	-	1-Octadecene	-
23.13	-	Pentadecanenitrile	-	-	Pentadecanenitrile	-
23.05	-	-	1-Heptadecene	1-Heptadecene	1-Heptadecene	1-Heptadecene
23.09	-	-	-	-	-	Benzenesulfonamide, N-butyl-
23.31	-	-	-	-	Octadecanal	-
23.31	-	-	-	Hexadecanal	-	Pentadecanenitrile
23.49	-	-	-	-	-	Propanetrione, diphenyl-
23.83	-	-	Pentadecanoic acid	-	Pentadecanoic acid	Pentadecanoic acid
24.06	-	-	-	-	Hexadecane, 1-chloro-	Hexadecane, 1-chloro-
24.20	-	-	-	1-Octadecene	-	-
24.32	Hexadecanenitrile	Hexadecanenitrile	Hexadecanenitrile	Hexadecanenitrile	Hexadecanenitrile	Hexadecanenitrile
24.47	-	-	Octadecanal	Octadecanal	Octadecanal	-
24.47	-	-	-	-	-	Hexadecanal
24.49	-	Pentadecanal-	-	-	-	-
24.53	-	-	-	-	-	(E,E)-7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene

24.54	-	-	-	Hexadecanoic acid, methyl ester	-	-
24.93	-	n-Hexadecanoic acid	n-Hexadecanoic acid	n-Hexadecanoic acid	n-Hexadecanoic acid	n-Hexadecanoic acid
25.21	-	-	-	-	1-Heneicosyl formate	-
25.32	-	-	-	Eicosane	-	-
25.39	-	-	-	Hexadecanenitrile	-	Heptadecanenitrile
25.52	-	-	-	-	Isopropyl Palmitate	-
25.98	-	-	-	Octadecanal	Octadecane, 1-chloro-	Octadecane, 1-chloro-
26.29	-	-	-	Heneicosane	Heneicosane	Eicosane
26.23	-	Octadecane, 1-chloro-	-	-	-	-
26.39	-	-	-	Heptadecanenitrile	Heptadecanenitrile	-
26.40	-	-	Octadecanenitrile	-	-	Octadecanenitrile
26.50	-	Heptadecanenitrile	-	Octadecanoic acid, methyl ester	-	-
26.93	-	-	-	-	Tetracontane	Hexadecanamide
26.92	-	-	Octadecanoic acid	Octadecanoic acid, 2-(2-hydroxyethoxy)ethyl ester	-	-

27.22	-	-	-	-	Tetratetracontane	-
27.25	-	-	Docosane	-	-	-
27.26	-	Heneicosane	-	Heneicosane	-	Heneicosane
27.81	[1,1':3',1"-Terphenyl]-2'-ol	[1,1':3',1"-Terphenyl]-2'-ol	[1,1':3',1"-Terphenyl]-2'-ol	[1,1':3',1"-Terphenyl]-2'-ol	[1,1':3',1"-Terphenyl]-2'-ol	[1,1':3',1"-Terphenyl]-2'-ol
28.17	-	Heneicosane	Heneicosane	Heneicosane	-	Heneicosane
28.47	-	-	-	-	-	Tetracontane
28.52	-	-	Tetracontane	Tetracontane	Tetracontane	Heneicosane
28.46	-	Tetracontane	-	-	-	-
29.14	-	Tetracosane	Heneicosane	Tetracosane	Heneicosane	-
29.52	-	Pentacosane	Heneicosane	Tetratetracontane	-	Docosane, 11-butyl-
29.88	Pentacosane	-	Heneicosane	Pentacosane	Pentacosane	-
30.14	-	-	-	Octacosane	-	Nonacosane
30.02	-	Nonacosane	-	-	Octacosane	-
30.39	-	Di-n-octyl phthalate	Triphenylphosphine oxide	Triphenylphosphine oxide	1,2-Benzenedicarboxylic acid, diisooctyl ester	Di-n-octyl phthalate
30.64	Pentacosane	Tetracontane	Heneicosane	Tetracontane	Heneicosane	1,2-Benzenedicarboxyli

						c acid, diisooctyl ester
30.81	-	Tetracontane	Tetratetracontane	Tetracontane	1-Hentetracontanol	Tetracosane, 11-decyl-
31.10	-	Tetratetracontane	Octacosane	Phosphine oxide, diphenyl(phenylmethyl)-	Heneicosane	Heneicosane
31.39	-	Nonacosane	-	Tetratetracontane	Heneicosane	Tetratetracontane
31.63	-	Pentacosane	-	Heptacosane	Pentacosane	Octacosane
31.66	-	Tetracontane	Tetracosane	Heneicosane	Tetracontane	Octacosane
32.04	Pentacosane	Nonacosane	-	Octacosane	Tetracontane	Tetracontane
32.34	-	-	Octacosane	Tetracosane, 11-decyl-	Tetracontane	Heneicosane
32.11	-	Tetratetracontane	Octacosane	Octacosane	-	Tetratetracontane
32.48	-	Tetracontane	Octacosane	Pentacosane	-	Tetracosane, 11-decyl-
32.65	-	Tetracontane	-	Tetracontane	-	Triacontane
32.96	-	Eicosane, 2-methyl-	-	Tetratetracontane	-	Squalene
33.08	-	Pentacosane	Octacosane	Tetratetracontane	-	Pentacosane
33.35	-	Tetracontane	Octacosane	Tetratetracontane	-	Pentacosane

33.53	-	Nonacosane	Pentacosane	-	-	Tetracontane
33.67	-	Nonacosane	-	-	-	Tetratetracontane
33.84	-	Nonacosane	-	-	-	Tetratetracontane
33.93	-	-	-	-	-	Octacosane