Summer 8-30-2018

Semiochemical Attractants of the Parasitic Fly Philornis Downsi in the Galapagos Islands

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SEMIOCHEMICAL ATTRACTANTS OF THE PARASITIC FLY *PHILORNIS DOWNSI* IN THE GALAPAGOS ISLANDS

by

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A thesis submitted in partial fulfillment
of the requirements for the
Doctor of Philosophy
State University of New York
College of Environmental Science and Forestry
Syracuse, New York August 2018

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ACKNOWLEDGMENTS

I thank the Galapagos Conservancy and the International Community Foundation (with a grant awarded by The Leona M. and Harry B. Helmsley Charitable Trust) who supported this research with funding for studies in the field and laboratory. In addition, this research would not have been possible without the support of the Galapagos National Park, Charles Darwin Foundation, and State University of New York, College of Environmental Science and Forestry.

I want to thank my advisor Dr. Stephen A. Teale. It has been an honor to be his student; with him I have built confidence and have learned so much. I hope we continue to work on future projects. I really appreciate all his contributions of time and ideas to make my Ph.D. experience productive and stimulating.

I thank the members of my Steering and Defense Committees. I give special thanks to Dr. Charlotte Causton who has been supporting me since 1999, and particularly now in this student stage she has been a strong inspiration and help in advice and working behind the scenes to coordinate the broader *Philornis* project including the chemical ecology work. For technical support in the laboratory I thank Dr. Katalin Boroczky and I especially want to thank Dr. Dong Cha for teaching new methodologies in GC-EAD and GC-MS, his contributions in my laboratory training have been invaluable. I also thank Dr. James Gibbs whose comments and observations put the focus of my work in perspective - looking beyond the lab to apply my results for conservation biology. I also want to thank Dr. Margaret Voss, without her support the last few years I would not have been able to move forward.

The members of the Teale lab, my friends and colleagues have contributed immensely to my personal and professional time at ESF. I am especially grateful for the

group Tian Xu, Laura Hansen, Kristin Dougherty, Hajar Faal and Elizabeth Semler with whom we started this adventure and Daniele Dryer who will also continue to work on *Philornis*. I thank Samantha Renda for conducting countless y-tube olfactometer assays of *P. downsi* extracts at CDF.

I thank my friends who were always supporting from different places helping with English (Lorraine Caputo), in the field (Paola Lahuatte, Andrea Cahuana, Erika Ramirez, Yazmin Cedillo and Sugey Caicedo), and technical assistance (Julio Delgado).

I thank my family for the love, moral support and good energy that they always have for me. Thank you so much Haydee, Ana Maria, Victor, Ariana, Scarlett, Maribel, Ma. Eugenia, Fernanda, Joseph, Carlos, Alberto, Carmen, Galo, Cecilia, Galo Efren, Paola, Families Ortega, Alvarez and Mendoza, and thanks to the family that I could choose my friends Alison, Lorena, Mateo, Javier R, Luis M, Luis O, Juan Carlo, Andres, Cristina, Margaret, Scott, Johanna, Nancy C, Ana Lucia, Jorge, Gabriela, Nancy WR, Bob and Colby.

Finally I want to thank and dedicate my thesis in memory to my father Enrique, he was so proud when I started with my PhD but he could not see me finish it, thanks old man.

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ABSTRACT

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Larvae of the parasitic fly *Philornis downsi* (Diptera: Muscidae) feed on the blood and tissues of passerine chicks. Parasitism by *P. downsi* contributes to increasing mortality and population declines in several bird species in the Galapagos Islands. This dissertation focuses on the identification of chemical attractants (food odors, sex or aggregation pheromones) for *P. downsi*. These attractants are important for increased trapping efficiency in the management of *P. downsi*. Dipteran mating and reproductive success are dependent on chemical communication, yet little is still known about the chemical ecology of most Diptera, with the exception of some agricultural pests and vectors of pathogens. My studies of chemical communication in *P. downsi* found some food odors, such as the volatile fermentation products (ethanol and acetic acid) produced by the yeast *Saccharomyces cerevisiae*, to be attractive. Maximal attraction was attained by a mixture of 3% ethanol and 0.3% acetic acid. The addition of 250 ml of this solution to an external reservoir that dispensed into a McPhail trap prolonged the effectiveness of these compounds in the field. Sex and aggregation pheromones are also important in this system; experiments identified males as the attractive sex. GC-EAD and GC-MS analyses on crude and photo-oxidized cuticular lipids of both sexes identified 18 photo-oxidation compounds produced by males as potential attractants for females. Genitalia extracts of each sex had markedly different volatile compounds. Extracts from male genitalia were significantly attractive to females in y-tube olfactometer assays ($p= 0.02$). Based on data presented here, I hypothesize that *P. downsi* mating begins with feeding behaviors, followed by location of host nests by male and female flies. Male flies then emit pheromones to attract the females. Mating occurs after mate location by visual identification and sexual or contact pheromones. Mating and communication systems in the calyptrate muscoids are poorly understood in general. In addition to identifying attractants specific to *P. downsi*, some of the work presented here may be broadly applicable to the chemical communication of muscoid flies in general.

Keywords: *Philornis downsi,* Galapagos*,* semiochemicals, avian parasite, muscoid mating systems

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CHAPTER I

INTRODUCTION

Chemical ecology is an interdisciplinary area of study that gained recognition when the first insect pheromone was identified by Butenandt (1959). Early studies addressed the chemistry of secondary plant metabolites and the ecological interactions of insects and plants (Ehrlich & Raven, 1964). This new discipline included the first studies of the chemical substances involved in the intra- and interspecific ecological interactions of living organisms (Eisner & Meinwald, 1995).

Chemical compounds or mixtures of chemical compounds that mediate intraspecific communication (pheromones) and interspecific communication (allelochemicals) are called semiochemicals (Litwack, 2010). Pheromones may mediate varied functions, including mate location, aggregation, species and sex recognition, and promotion or inhibition of copulation. Allelochemicals can also mediate many functions, such as searching for food, recognition of nesting sites, and chemical mimicry (Blomquist et al.,1993).

Insects have evolved sophisticated chemical communication systems that regulate much of their behavior. These systems have two components: production of highly specific chemical signals and similarly specific receptor systems, which enable the organism to discriminate between a meaningful signal and background noise (Afonso-Carrillo, 2012).

The early studies focused mainly on priority species of economic importance. Thus, agricultural and forest pests, primarily beetles and moths, have received the most

attention. Due to the large number of pests of agriculture and forestry in the Lepidoptera, significant effort has been spent on the structural elucidation of long-range female sex pheromones in this group. As of 2004, pheromones of more than 530 species of moths had been identified (Schulz, 2004).

The first chemical identification of a pheromone was that of a moth, the female silkworm, *Bombyx mori* (Butenandt, 1959). Efforts to elucidate this pheromone, "Bombykol," required about 20 years and at least half a million female abdomens (Blomquist & Vogt, 2003). The second insect pheromone identification was that of a bark beetle and was reported in 1966 (Silverstein et al., 1966). Subsequently, the identification of female moth sex pheromones accelerated, with more than 40 reported by 1976 (Shorey, 1976).

Coleoptera is the largest insect order and includes many agricultural and forest pests. It also has been the subject of much chemical ecological research (Cardé & Bell, 1995), including the elucidation of cuticular hydrocarbons as pheromones (Schulz, 2004; Blomquist & Bagneres, 2010), and aggregation and anti-aggregation pheromones (Jacobson, 1972; Cardé & Bell, 1995), among others.

Studies of chemical ecology have also focused on Diptera, another large order of great economic importance. Many species of flies are significant agricultural pests, or are vectors of human or animal pathogens (Resh & Cardé, 2009). The first dipteran sex pheromone reported, (Z)-9-tricosene, was identified by Rogoff et al. (1964) in the common house fly, *Musca domestica*, and the first study of mating behavior in flies was in 1911. In that study, experiments were conducted to determine sex recognition and sexual selection problems in Drosophila (Sturtevant, 1915). During the 1970s,

pheromones were identified in the tsetse fly, *Glossina morsitans* (Carlson et al., 1978). More recently, the chemical components of pheromones have been elucidated in other species such as *D. melanogaster* (Jacobson, 1972; Scott & Richmond, 1987; Bartelt et al., 1988; Howse et al., 1998).

In muscoids like other flies, the ecological basis of mating systems and reproductive success is universally correlated with the success of food foraging (sugar or blood), ecological conditions and body size, and chemical communication. In addition, the morphology of the species has a strong influence on the evolutionary processes of sexual selection and sexual conflicts (Blum & Blum, 2012).

Very little is known about the mating system of the bird parasite, *Philornis downsi*, a species that has invaded the Galapagos Islands (Kleindorfer & Dudaniec, 2016; Fessl et al., 2018). This information is crucial to understanding its reproductive biology and for finding control methods to reduce its impacts on Darwin's finches and other endemic birds in the archipelago (Causton et al., 2013; Fessl et al., 2018). To better understand the mating system of *P. downsi*, as a first step I will review the factors known to influence mating systems in other higher Diptera, in particular calyptrate muscoid parasites including mate searching, resource defense, aerial swarming, development and maturation of eggs, host selection, and pheromone production.

Dipteran mating systems

Comparative studies of mating system diversity across related taxa are useful for guiding research on the roles of chemical signals in poorly known species such as *P. downsi*. Many complex reproductive behaviors have been described in the Diptera.

Among these, associations between the distribution of resources including food and oviposition substrates, and mating location are common. This complexity arises because flies frequently do not conform to typical simple reproductive systems, but rather have diversified their reproductive strategies. Pitnick et al., (1999) and Presgraves et al., (1999) suggested that male and female reproductive traits show correlated evolutionary change.

On the other hand, some mating related behaviors have evolved very rapidly among groups of closely related flies. For example, differences have been reported in precopulatory courtship, duration of fights between males and duration of copulation, and these differences in the mating system can influence the rate of speciation (Hoikkala et al., 1994; Bonduriansky & Brooks, 1999; Chen et al., 2002; Barbosa, 2011).

Reproductive behavior of Calyptrate muscoid parasitoids

Evolutionary diversity in different ecological systems, have positioned flies as model organisms to understand mating systems. According to Emlen & Oriing (1977), mating systems in dipterans are influenced by physical and environmental factors of the ecosystem (Yeates & Weigmann, 2005). Fly reproductive behavior can be viewed in four stages: search for available partners, precopulatory behavior, copulation and post-copulatory behavior. Additionally, reproductive success involves morphological, behavioral, physiological, or biochemical factors. In this sense, patterns in sexual selection affect the morphology and behavior of males and females that adopt the norms at each encounter site or host (Wilkinson & Johns, 2005).

Mate searching.

Strategies for locating ephemeral resources -- food and sexual partners -- have been studied in flesh flies (Sarcophagidae) and blow flies (Calliphoridae). Experiments indicated that sarcophagids tended to arrive to a resource before the calliphorids and the smaller individuals were the last to leave the aggregation sites. In addition, experiments showed that olfactory and anemotactic orientation (movement in response to air currents) used together are more efficient in pinpointing the resource's location. (Spivak et al., 1991).

Such resource-seeking behavior was observed in the screwworm, *Cochliomyia hominivorax* (Calliphoridae). Mackley & Long (1983) determined that the activity of this fly in a given plant species seems to be related to flowering patterns and possibly to the color of the flower. The observed flies appeared to gather, feed and mate in flowering vegetation up to five days after eclosing, and then dispersed or died. However, no fly activity was observed when the plants were not in flower, except in certain places where the males were waiting for the females in the vegetation near blossoms.

Mate-searching in the hymenopteran brood parasite, *Miltogramma rectangularis* (Sarcophagidae), suggests that males come from the host nesting site or along its border, regularly changing perch sites, flying very close to the ground in an irregular zigzag, and often turning towards small pebbles or twigs. When two males encounter, they seem to rotate one around the other before advancing and landing next to the adult females. Most females were receptive for a single copulation, suggesting that the search for virgin females benefits males by favoring reproduction (Alcock, 2000).

In tachinids several mating strategies have been observed. In many species, mate-searching is strongly associated with the antennae and the strategy of waiting for females. These observations indicate that in males, the frons is generally narrower and the eyes larger than the females. Males wait in a particular place, which is usually leaves, bare twigs, or tree trunks. Several congeners may wait or fly close to each other, and often spend much time chasing each other (O'Hara, 1996; Stireman, O'Hara, & Wood, 2006). A different type of mate-searching behavior in tachinids suggests that males visit sites with hosts and inspect them for females, which wait until the male has decided that it is a good site for mating (O'Hara, 1996). In another mate-searching strategy observed in tachinids including *Therobia leonidei*, males and females search for hosts and mates, flying over and locating the sounds of female grasshoppers (*Poecilimon thessalicus*). This acoustic resonance search behavior has also been observed in other tachinids of the genus *Ormia* (Lehmann et al., 2001; Lakes-Harlan & de Vries, 2014).

In female *Hypoderma tarandi* (Oestridae), individual flight ability may be very important for reproductive success, because they (females) must fly hundreds of kilometers in search of their host and a mate. As a strategy, the males dominate an area for mating, and the females select a dominant male (Anderson et al., 1994).

Resource defense.

Finding and choosing mates influences the evolution and sexual dimorphism of species. Many insect groups, including flies, often develop specific traits or characteristics that enhance their ability to obtain and defend resources and hence

attract mates; for example, the males of a species tend to have larger bodies, which allows them to attract and defend more females (Ding & Blanckenhorn, 2002). Resources that attract multiple females, such as oviposition or feeding sites, provide an opportunity for mating, and mating systems are typically associated with resource advocacy with pre-copulation mating activity (Burk, 1981).

In stable flies, *Stomoxys spp*. (Muscidae), the defense of a resource (e.g. a host) has been observed in the field. At the same time, the host also presents a defensive behavior against the attack of this fly. It was also observed that young flies often spend much time defending the resource against adult flies and other competitors, which decreases the reproductive success of young flies (Schofield & Torr, 2002).

Stable flies prefer to feed on adult cows, because calves show higher rates of defensive behavior against biting flies and discourage flies from feeding. Other competitors, such as the tsetse fly, produce aggregation pheromones on a host, to inhibit other parasitic flies, which apparently reduce the feeding of stable flies. In this sense, the defensive behavior of an individual stable fly would be determined by its odor and the host's age, as well as by the chemical defenses of competitors (Torr et al., 2006).

Aerial swarming.

Aerial swarms are one of the most frequent mating behaviors historically documented in Diptera. These swarms generally contain from 10 to 1,000 individuals grouped within a few cubic meters on a visible, generally high landmark such as a ridge, a treetop, a fence post, or even free-floating vegetative mats on the surface of the

ocean. These aerial swarms are generally dominated by males, although a few examples include swarms of female flies (Wilkinson & Johns, 2005).

Observations of *Stomoxys calcitrans* indicate that they tend to create swarms near livestock, on vertical surfaces, and near light-colored materials. The males of this species rest on waiting stations and then carry out flyovers involving aerial interactions in the swarm. Receptive females were also found grouped in waiting stations and then copulate with males in the air or on the ground. This behavior of swarming and copulation occurs in sunny places in winter and under shade in summer (Buschman & Patterson, 1981).

Swarming behavior of *Ophyra leucostoma* (Muscidae) involves males hovering under tree branches and tall shrubs. This stationary flight is frequently interrupted by short pursuits after small flying insects. Additionally, it has been observed that fast circular flights and short chases with no apparent objective occur when other males are nearby. Longer chases extend to several meters from the swarm site and have been observed when other flying insects approach. Swarming behavior in this species closely resembles territorial defense behavior (Pajunen, 1982).

The role of aerial swarming in courtship and mating has also been studied in *Lispe spp*. (Muscidae) in which both sexes perform circular and lateral flights separated by regular time intervals. Once landing at a meeting point, a mature adult male performs a dance as it approaches the female while young males surround them and fiercely fight for a role in the courtship (Frantsevich & Gorb, 2006).

Observations in the housefly *Fannia canicularis* (Muscidae) indicate that males establish a set of lek-type mating aggregations beneath reference points on vegetation

in order to intercept approaching females. The position of the male patrol stations relative to the reference point suggests that females approach the reference points from the side and cross the dorsal visual field of patrolling males. Males usually approach the reference point from below and, in the absence of other flies, patrol the airspace near the reference point (Zeil, 1986).

Host selection.

In parasitic muscoids, studies on the feeding and reproductive behavior that involves searching for hosts and chemical ecology have focused on tachinids. One of the interesting features of the Tachinidae is their diversity of oviposition strategies, which may include detection and selection of the host through the use of acoustic cues, and direct and indirect parasitization (Nakamura et al., 2013).

Field observations of *Ormia ochracea* (Tachinidae) females indicated that these flies detect the songs of the cricket *Gryllus lineaticeps* which vary in chirp frequency, chirp duration, or both, but not in amplitude. *Ormia ochracea* preferred to approach songs with higher chirping rates and longer chirp durations (Wagner & Basolo, 2007).

The evolution of host selection has been studied in *Emblemasoma auditrix* (Sarcophagidae). In this species, the acoustic senses are specialized in the detection and parasitization of cicada (*Okanagana rimosa*) males. The observations of this species suggest very complex evolutionary processes because although the auditory capacity of *E. auditrix* does not reach the frequencies produced by the cicadas, the flies apparently use a series of adaptations to find their host. The auditory senses of the fly

are sensitive to substrate vibrations to which the hearing is fine-tuned (Lakes-Harlan et al., 2014).

Reproductive behavior in relation to host choice has been investigated in the horn fly, *Haematobia irritans* (Muscidae), which is an obligate hematophagous parasite of cattle. Observations in the field showed that these flies initially feed on blood from a healthy cow and then move to a lower part of the cow's body, waiting to see when the cow defecates. This action triggers an immediate reaction of the fly, which deposits its eggs in the fresh manure; this seems to suggest that the choice of the place to lay the eggs depends on the freshness of the manure (Kuramochi, 2000).

Similar behavior in terms of breeding site preferences and feeding strategies was also documented in manure-feeding calliphorid and sarcophagid larvae. These observations indicated that the selection of feeding site is optimally suited for larval development as determined by the hardness of the manure crust (Archer & Elgar, 2003).

An investigation of host selection behavior in the hematophagous bird parasite *Protocalliphora sp*. (Calliphoridae) determined that host selection was strongly influenced by colony size of nesting birds, nest proximity, multi-year nesting in the same site, as well as nest size. Older, larger, and more frequently occupied nests were more often infested by these flies (Bennett & Whitworth, 1992).

Environmental conditions such as wind and temperature are important factors in host selection. For example, it has been observed that climatic factors affect the activity of the reindeer warble fly, *Hypoderma tarandi* (Oestridae) which is more active on warm

sunny days (15-22°C) with little or no wind. Days with these characteristics were more favorable for host-searching and for mating flight activities (Anderson et al., 1994).

Pheromone production.

The Diptera have developed diverse courtship strategies and in many cases sex pheromones have been shown to be involved in these processes. Yet, before the 1960s, little was known about the chemical and biological importance of hydrocarbons (the chief component of pheromones) in the behavior of insects and other arthropods. Therefore, studies on the structure of hydrocarbons were not of great interest. Since then, hydrocarbon studies have identified a variety of behavioral roles, particularly in chemical communication (Blomquist & Bagneres 2010).

Chemoreception, age and nutritional status of individuals play an important role in regulating the mating behavior of many Diptera. Additionally, some semiochemicals are behavioral primers affecting hormonal function in the receiving individual (Jang, 2002). Although pheromones are an important component of courtship they are but one of many types of signals used by the insect, and in most families of Diptera, the functionally active components are not known (Wicker-Thomas, 2007).

A wide range of functions has been documented in dipteran behavior (dominance of aggregation, courtship, mate discrimination) and reproduction (fecundity, sex ratio), associated with the role of pheromones in cuticular hydrocarbons (Blomquist & Vogt, 2003; Blomquist & Bagneres, 2010). The known sex pheromones in dipterans are mainly low volatility and non-volatile molecules that are detected over short distances or upon contact during courtship (Wyatt, 2003).

In some species of flies, pheromones have an effective range of only a few centimeters, and trigger courtship behavior or increase mating ‘attack’ activity, as in *Musca domestica* (Prestwich & Blomquist, 1987). For example, the female screwworm fly, *Cochliomyia hominivorax,* produces contact pheromones which are detected by males and which function as a pre-copulatory stimulus (Hammack, 1986).

Laboratory observations of *M. domestica* showed that the females emit low attraction volatiles for both males and females. The results of these tests indicated that young (one-day-old) virgin females attracted few or no males, but older (seven-day-old) virgin females, alive or freshly killed, consistently attracted virgin males. Virgin males often tried to mate with other young males (Murvosh et al., 1964). Similar behaviors between young males have also been recorded during captive breeding trials with *Philornis downsi* (Lincango com. pers., 2014).

Philornis downsi

Chemical ecology studies of *Philornis downsi*, especially those addressing attractants, are important because the results can be useful for the monitoring and capture of this species using traps (Causton et al., 2013). Preliminary studies suggest that *P. downsi* can produce a pheromone to attract mates, and that flies are attracted by fermentation products and odors produced by the breakdown of proteins (Muth, 2007; Lincango & Causton, 2009; Collignon, 2011).

The metabolic processes of fermentation and protein degradation are of particular interest in that a wide range of potential attractants, including acetic acid, ethanol, many different amino acids and α-keto acids, are produced.

Capture methods using potential attractants in various types of traps have been tested in the past to evaluate viable alternatives for controlling *P. downsi* populations in the short term, and to assess the success of other management strategies. The results of these studies suggest that sticky cylindrical traps are less effective than McPhail traps in the capture of adult *P. downsi* individuals; horizontal, flat adhesive traps are not effective at all (Doherty, 2012; Hellman & Fierke, 2009).

The analysis of potential attractants like BioLure, milk, papaya, amine, putrescine, methylamine, indole and (*Z*)-9-tricosene, indicated that these potential attractants were not effective either (Lincango & Causton, 2009).

Collignon (2011) investigated sexual dimorphism in *P. downsi* cuticular hydrocarbons. His results suggest that males could potentially produce volatile pheromones by photo- and auto-oxidation of cuticle hydrocarbon lipids. Evidence suggests that females are unlikely to produce long-range pheromones because of the low volatility of their cuticular hydrocarbon components, which are more likely contact pheromones.

Additional experiments are required to identify potential attractants of *P. downsi* (nest and/or food odors) as well as determine the role of semiochemicals in mating and oviposition. Deciphering the chemical communication system of *P. downsi* and its hosts will provide new insights on the behavior and ecology of these insects.

It may also lead to the development of control and monitoring tactics for this invasive fly for the benefit of the conservation of Galapagos land birds. Understanding the communication system of *P. downsi* is an important factor in developing techniques

to disrupt mating and ultimately develop a management plan to control the fly population.

CHAPTER II

Identification and optimization of microbial attractants for *Philornis downsi*, an invasive fly parasitic on Galápagos birds

J Chem Ecol. 2016 Nov; 42(11):1101-1111. Epub 2016 Oct 15.

ABSTRACT

We investigated the role of olfactory cues from actively fermenting yeast (*Saccharomyces cerevisiae*) in attraction of adult *Philornis downsi* and identified two synergistically attractive yeast volatiles. Larvae of this invasive fly parasitize the hatchlings of passerines and threaten the Galapagos avifauna. Gas chromatography coupled with electroantennographic detection (GCEAD), coupled gas chromatography-mass spectrometry (GCMS), and field trapping experiments were used to identify volatile compounds from a yeast-sugar solution. EAD responses were consistently elicited by 14 yeast volatiles. In a series of field trapping experiments, a mixture of the 14 EAD-active compounds was similarly attractive to *P. downsi* when compared to the yeast sugar solution, and we found that acetic acid and ethanol were essential for attraction. A mixture of 0.03 % acetic acid and 3 % ethanol was as attractive as the 14-component blend, but was not as attractive as the yeast-sugar solution. *Philornis downsi* showed positive and negative dose-responses to acetic acid in the ranges of 0.01 ~ 0.3 % and 0.3 ~ 9 %, respectively. Further optimization showed that the mixture of 1 % acetic acid and 3 % ethanol was as attractive as the yeast-sugar solution. Both mixtures of acetic acid and ethanol were more selective than the yeast-sugar solution in terms of non-target moths and *Polistes versicolor* wasps captured. These results indicate that acetic acid and ethanol produced by yeasts are crucial for *P. downsi* attraction to fermented materials on which they feed as adults and can be used to manage this invasive fly in Galapagos.

INTRODUCTION

Philornis downsi Dodge & Aitken (Diptera: Muscidae) is an avian parasite that is native to South America with records from Trinidad and Tobago (Dodge & Aitken, 1968), Brazil (Mendonça & Couri, 1999), Argentina (Silvestri et al., 2011), and mainland Ecuador (Bulgarella et al., 2015). Although the adults are fruit feeders, the female flies lay eggs in bird nests, and the hematophagous larvae feed on the blood of bird hatchlings, reducing growth and causing high levels of mortality (Fessl et al., 2006; Kleindorfer & Dudaniec, 2016).

Through an unknown pathway, this fly has invaded the Galapagos Islands, where parasitism by *P. downsi* has been linked to recent declines in endemic passerine birds including species of Darwin's finches and species that are under threat of extinction (Cimadom et al., 2014; Cunninghame et al., 2015; Kleindorfer & Dudaniec, 2016; O'Connor et al., 2010).

Philornis downsi infestation on Darwin's finches was first reported in 1997 (Fessl et al., 2001), but a 1964 specimen in the insect collection of the California Academy of Sciences suggests it was introduced to the Galapagos by that date or earlier (Causton et al., 2006). Larvae of *P. downsi* attack at least 16 endemic passerine birds in the Galapagos during the breeding season, and are recognized as a serious obstacle to the conservation of Darwin's finches (Causton et al., 2013; Koop et al., 2011).

The likelihood of extinction of one or more species of these birds is exacerbated by their small population sizes (Cunninghame et al., 2015; Koop et al., 2016). Various management tools are currently in development to reduce the negative impacts of *P.*

downsi on Darwin's finches including biological control (Bulgarella et al., 2015), nest autofumigation (Knutie et al., 2014), and artificial rearing for use in the sterile insect technique (Causton et al., 2013; Lahuatte et al., 2016). Koop et al. (2016) suggest that even modest *P. downsi* mitigation may increase host survival sufficiently to prevent extinction.

Recent studies have demonstrated the pervasive role of microbial volatiles in insect attraction (reviewed in Davis et al. 2013). In some insect systems, such as *Drosophila melanogaster* and the codling moth, *Cydia pomonella*, it has been suggested that the attraction of these insects to fruit is actually mediated by volatiles produced by yeasts living on the fruit phyllosphere (Becher et al., 2012; Witzgall et al., 2012).

It is well known that fermentation volatiles attract a wide range of insects, such as moths, wasps, flies, and natural enemies in nature (Landolt et al., 2014; Thomas, 2003; Yamazaki, 1998), and recently yeast volatiles have been used successfully to develop a strong synthetic attractant to control an important invasive agricultural pest, *Drosophila suzukii* (Cha et al., 2012 b, 2014a, b). Thus, our goal is to understand chemical signals involved in the attraction of adult *P. downsi* to yeast fermentation volatiles to ultimately develop a synthetic chemical attractant that can be used in population monitoring and suppression.

It has been shown that papaya juice becomes gradually more attractive to *P. downsi* in Galapagos after aging for four to five days, suggesting that *P. downsi* is attracted to yeast or other fermentation volatiles (Lincango & Causton 2009). This hypothesis has not been tested and any chemicals involved have not been studied.

Attractive fermentation -or food- based baits may be effective for monitoring and controlling *P. downsi*. However, the attractiveness of food-baits generally is ephemeral so they must be replaced frequently. In addition, fermentation food baits are well known for their broad attractiveness to diverse insect groups (see references in Cha et al. 2015), including muscid flies (Cha et al., 2013, 2015; Landolt et al., 2015).

A recent study shows that a synthetic chemical lure that is composed of a small subset of chemicals released from fermentation baits is more selective for target insects than the fermentation bait (Cha et al., 2015). Therefore, synthetic chemical lures can be more selective, and once the active behavior modifying chemicals are identified, chemical dispenser(s) and chemical release rate(s) can be optimized to prolong the attractiveness of the lure in the field.

Currently, there is no synthetic attractant for *P. downsi* that is as effective as or more selective than food baits. Here, we report on two key attractant volatiles that synergistically attract *P. downsi* to actively fermenting yeast-sugar solutions. Specifically, we: 1) identified yeast volatile compounds that could be detected by *P. downsi* antennae, 2) evaluated the attractiveness of various chemical blends in a series of field trapping experiments, 3) determined a key set of yeast fermentation volatiles that were required for *P. downsi* attraction, and 4) optimized the dose of the two-component mixture so that it was as attractive as the original yeast-sugar solution.

We also examined whether the two-component synthetic chemical lure based on yeast-sugar fermentation volatiles was more selective for non-target insects than yeast-sugar solution.

METHODS AND MATERIALS

Insects Material *Philornis downsi* larvae and pupae were removed from fledged and failed nests of the small tree finch (*Camarhynchus parvulus*) and the warbler finch (*Certhidae olivacea*) in the remnant of the Scalesia forest near 'Los Gemelos' in the humid highlands (400–700 m a.s.l.) of Santa Cruz Island (0°37′ 34″ S, 90°23′ 10″ W).

Adults were field-trapped near Puerto Ayora of Santa Cruz Island, Galapagos, under permit from the Galapagos National Park Directorate, and shipped or hand-carried to the quarantine laboratory at the State University of New York, College of Environmental Science and Forestry (SUNY-ESF), Syracuse, NY, USA.

Eclosed adult flies were grouped by sex (4–5 flies/4 l cage), and kept in a growth chamber (Percival Scientific, Inc., Perry, IA, USA) at 23.0 °C, 70 % RH, 16:8 L:D on blackberry puree, brown sugar, water, and powdered milk prior to testing. Adult flies at 0–10-d-old were used for GCEAD analyses.

Field Trapping All field experiments were conducted on Santa Cruz Island, Galapagos, at El Barranco (0°44′34.1″S, 90°18′10.4″W, elevation 15–41 m m) - an arid, lowland area predominantly composed of Opuntia and *Jasminocerus cacti*, and *Cordia lutea*, *Acacia sp*., and *Parkinsonia aculeata* trees.

The plastic McPhail-type trap (Naturquim, Guayaquil, Ecuador) was used for field trapping. In this, the bottom third of the trap is yellow and the top two-thirds is clear with a 5-cm diam bottom entry hole for attracted insects. The trap bottom holds up to 300 ml of drowning solution.

Each trap was baited with 150 ml of drowning solution containing attractants as described below. For each field experiment, a randomized complete block design was used with 10 to 30 replicates. Traps were placed at a height of approximately 4 m, and were no less than 10 m apart.

Female and male *P. downsi* were counted every 24 h during the trapping period. Previously, water controls consistently attracted zero *P. downsi* (Lincango & Causton, 2009), so we did not include negative controls in our experiments. Traps and drowning solutions were not replaced during the 2–3 d trapping period. After each experiment, traps were washed with a strong, unscented detergent and exposed to direct sunlight for at least 5 d.

Volatile Collections Headspace volatiles were collected from fermenting yeast-sugar solution by using 2.4 L closed volatile collection chambers (ARS, Inc., Gainesville, FL, USA; Glass shop, Cornell University, Ithaca, NY, USA) with one air inlet adapter (7 mm ID) on the top and an outlet adapter (7 mm ID) on the bottom wall.

Yeast-sugar solution was prepared by adding baker's yeast (Saccharomyces cerevisiae, 2 g; Levapan, Quito, Ecuador) to a solution of sugar (10 g) in distilled water (200 ml) at 30–35 °C, and then immediately put into a collection chamber. Clean air from a filtering and delivery system (ARS Inc., Gainesville, FL, USA) was pushed into the chamber at 0.75 L min−1 through the top inlet port, and volatiles from yeast solution were pushed out through activated charcoal sorbent tubes (ORBO32-small, Supelco Inc., Bellefonte, PA, USA) on the bottom of the chamber.

An additional ORBO filter was used to ensure that no breakthrough of volatiles from the adsorbent filter occurred. Adsorbent collections were made over a 3 d period at room temperature using the same volatile trap (24.2 ± 0.5 °C, 16 L:8D). Volatiles were eluted with 500 µl methylene chloride every 24 h from the filters of a given sample and combined across the three collecting days. The extracts were kept at −20 °C and subjected to GC-EAD and GC-MS analyses.

Coupled Gas Chromatography-Electroantennographic Detection (GC-EAD)

Analyses

Coupled GC-EAD analyses were performed using an Agilent 5890 Series II gas chromatograph equipped with a DB-Wax capillary column (30 m × 0.25 mm ID, 0.25 µm film thickness; Agilent Technologies, Wilmington, DE, USA) or an HP-5 capillary column (30 m × 0.25 mm ID, 0.25 µm film thickness; Agilent Technologies) in splitless mode with 1 min sampling. The oven temperature was programmed for 5 min at 40 °C, 15 °C/min increase to 250 °C, and then held for 5 min. Injector temperature was set at 250 °C. Helium gas was the carrier at a constant flow rate of 2 ml/min. The column effluent was split 1:1 in the oven via an Y-splitter (Supelco, Bellafonte, PA, USA) with nitrogen added as a make-up gas (8 ml/min) using another Y-splitter. One arm of the splitter led to the flame ionization detector (FID) (280 °C) and the other to the heated EAD port (280 °C) introduced into a cooled humidified air stream (1 L/min) directed toward the antennae of the mounted fly.

Whole head preparations were made of individual flies, age 3–12 d, for GC-EAD analysis, as described previously from similar studies with other species of flies (Cha et al. 2011a, b, 2012a, b). The head was excised and its antennae positioned between two gold wire electrodes immersed in saline-filled (Drosophila ringer solution; 46 mmol NaCl, 182 mmol KCl, 3 mmol CaCl2) micropipettes in an acrylic holder.

The output signal from the antennae was amplified (10×) by a custom high input impedance DC amplifier and recorded on an HP 3393A integrator. For the GC-EAD analysis of yeast volatiles, a total of 19 different antennal pairs from 11 female and 8 male flies (2–9 replicated runs/pair) were tested on adsorbent extracts and SPME samples.

Chemical Analyses

Coupled gas chromatography-mass spectrometry (GC-MS) analyses were carried out with an Agilent 5975C MSD coupled with an Agilent 7890GC equipped with a DB-WAX capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness; Agilent Technologies) or an HP-5 capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness; Agilent Technologies). Carrier gas was helium (1.0 ml/min constant flow). The GC temperature program was the same as used for the GC-EAD analyses.

The MS transfer line was held at 250 °C for DB-WAX or 280 °C for HP-5 ms columns; the MS source was 230 °C; the MS quad was 150 °C. Mass spectra were taken in EI mode (at 70 eV) in the range from 40 m/z to 350 m/z with a scanning rate of 2.36 scan/s. GC-MS data were processed with the MDS-Chemstation software (Agilent Technologies).

Volatile compounds that were consistently EAD active were tentatively identified by comparison of chromatographic retention times and mass spectra with the NIST 2.0 mass spectra database and with those of authentic standards analyzed on the same instrument. To confirm the identities of EAD-active compounds, authentic standards (10 ng/µl) were tested on *P. downsi* antennae and co-injected with the yeast sugar adsorbent extracts for EAD response.

The release rate (mg/day/150 ml yeast-sugar solution) and relative ratio (%) of the compounds (Table 1) that elicited EAD responses was derived from the adsorbent collection. For quantification, blends of standard compounds containing 0.1, 1, 10, or 100 ng each of all EAD-active compounds in 1 µl of methylene chloride were prepared and analyzed to obtain calibration curves ($r^2 > 0.99$ for all 12 compounds).

The loading amounts of different EAD-active compounds for trapping experiments were calculated according to the relative ratio and so the total amount of 12 EAD-active chemicals (excluding acetic acid and ethanol) in trap drowning solution was approximately 1 µg/µl (Table 1), which resulted in similar GC profiles to yeast-sugar solution.

Estimated concentrations of acetic acid (0.03 %) and ethanol (3 %) in yeast-sugar solution (e.g., Banat et al. 1998; Erasmus et al. 2004) were used until we experimentally optimized the concentration of acetic acid in Experiment 6 and ethanol in Experiment 7.

Chemicals

Ethyl butyrate (99 %, CAS No. 105–54-4), 2-methylbutyl acetate (99 %, CAS No. 624–41-9), 3-methylbutyl acetate (98 %, CAS No. 123–92-2), 2-methyl-1-butanol (99 %, CAS No. 137–32-6), 3-methyl-1-butanol (98 %, CAS No. 123–51-3), ethyl hexanoate (99 %, CAS No. 123–66-0), 2-phenylethyl acetate (99 %, CAS No. 103–45-7), and 2-phenylethanol (≥ 99 %, CAS No. 60–12-8) were purchased from TCI America (Portland, OR, USA).

Isobutanol (2-methylpropan-1-ol) (99 %, CAS No. 78–83-1), 1-propanol (99 %, CAS No. 71–23-8), 3-hydroxybutan-2-one [acetoin] (≥ 96 %, CAS No. 513–86-0), and 2,3-butanediol (98 %, CAS No. 513–85-9) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol (200 proof, CAS No. 64–47-5) and acetic acid (99.8 %, CAS No. 64–19-7) were purchased from Pharmco (Brookfield, CT, USA) and Fisher Scientific (Pittsburgh, PA, USA), respectively.

Trapping Experiments

Experiment 1:

Yeast vs. Six-Component Blend (6c) vs. 14-Component Blend (14c) The objective of this experiment was to determine whether two different EAD-active compound synthetic blends are as attractive to *P. downsi* as the yeast-sugar solution. Traps were baited with (i) yeast-sugar solution, (ii) six-component blend (6c) composed of the six most abundant EAD-active compounds, and (iii) 14-component blend (14c)

composed of all 14 EAD-active compounds (see Table 1 for chemical composition of synthetic blends).

Yeast-sugar solution was prepared by dissolving 75 g sugar in 1500 ml water (30–35 °C) then mixing in 15 g baker's yeast (Levapan del Ecuador, S.A., Quito, Ecuador). Three temporal replicates were carried out during 15–18 October 2015, 24–27 November 2015, and 12–15 January 2016 due to fluctuations in the population of *P. downsi*, with 10 replicated blocks each (N = 30) maintained for 3 d during each temporal replicate.

Experiment 2:

14-Component Blend (14c) vs. 13-Component Blend (13c) vs. Seven-Component Blend (7c) In Experiment 1, blend 6c was not as attractive as yeast, and blend 14c was as attractive as yeast, suggesting that some or all of the eight volatile compounds not included in 6c (see Table 1) were responsible for the difference.

The objective of this experiment was to determine whether the difference in the attractiveness of 6c and 14c was due to acetic acid or to any of the other 7 minor EAD-active compounds. Traps were baited with (i) 14c, (ii) 13c (6c plus seven minor EAD active compounds: testing the effect of seven minor EADactive compounds), and (iii) 7c (6c plus acetic acid: testing the effect of acetic acid) (Table 1). Ten replicated blocks were maintained for 3 d between 21 and 24 January 2016.

Experiment 3:

Seven-Component Blend (7c) Subtraction Test The objective of this experiment was to determine which components of the 7c blend, in addition to acetic acid (tested in experiment 2), are key to attraction of *P. downsi*. Thus, we baited traps with (i) 7c, (ii) 7c minus ethanol, (iii) 7c minus acetoin, (iv) 7c minus isobutanol, (v) 7c minus 3-methyl-1-butanol, (vi) 7 c minus 2-methyl-1-butanol, and (vii) 7c minus 2-phenylethanol (Table 1).

Two temporal replicates consisting of two trapping experiments during 2–5 February 2016 and 7-10 February 2016 were carried out with seven replicated blocks (N = 14) maintained for 3 d during each temporal replicate.

Experiment 4:

Acetic Acid + Ethanol vs. Seven-Component Blend (7c) The objective of this experiment was to determine whether a mixture of acetic acid and ethanol would be as attractive as the 7c blend. We baited traps with (i) 0.03 % acetic acid +3 % ethanol, and (ii) 7c. Twenty replicated blocks were maintained for 3 d between 23 and 26 February 2016.

Experiment 5:

Yeast vs. Acetic Acid + Ethanol The objective of this study was to determine whether a mixture of acetic acid and ethanol would be as attractive as yeast-sugar solution. We baited traps with (i) 0.03 % acetic acid +3 % ethanol, and (ii) yeast-sugar solution. Twenty replicated blocks were maintained for 3 d between 1 and 4 March 2016.

Experiment 6:

Acetic Acid Optimization The objective of this experiment was to determine the concentration of acetic acid that would be most attractive to *P. downsi* in a mixture with 3 % ethanol. A range of acetic acid concentrations was manipulated in two experiments. In the lower range acetic acid experiment, we baited traps with (i) 0.01 % acetic acid +3 % ethanol, (ii) 0.03 % acetic acid +3 % ethanol, (iii) 0. 1 % acetic acid +3 % ethanol, and (iv) 0.3 % acetic acid +3 % ethanol. In the higher range acetic acid experiment, we baited traps with (i) 0.3 % acetic acid +3 % ethanol, (ii) 1 % acetic acid +3 % ethanol, (iii) 3 % acetic acid +3 % ethanol, and (iv) 9 % acetic acid +3 % ethanol. For the lower range experiment, there were two temporal replicates consisting of two trapping experiments during 18–20 March 2016 and 20–22 March 2016. For each, 10 replicated blocks (N = 20) were maintained for 2 d. For the higher range experiment, 10 replicated blocks were maintained for 3 d between 9 and 12 March 2016.

Experiment 7:

Ethanol Optimization The objective of this experiment was to determine the concentration of ethanol that would be most attractive to *P. downsi* in a mixture with 1 % acetic acid. For this experiment, we baited traps with (i) 1 % acetic acid +0.3 % ethanol, (ii) 1 % acetic acid +3 % ethanol, (iii) 1 % acetic acid +9 % ethanol, and (iv) 1 % acetic acid +15 % ethanol. There were two temporal replicates consisting of two trapping experiments during 14–16 March 2016 and 16–18 March 2016. For each, 10 replicated blocks (N = 20) were maintained for 2 d.

Experiment 8:

Optimized Acetic Acid + Ethanol vs. Yeast This experiment was similar to Experiment 5 but used the optimized concentration of acetic acid and ethanol from Experiments 6 and 7, respectively. The objective was to determine whether a mixture of optimized amount of acetic acid and ethanol would be as attractive as yeast-sugar solution to *P. downsi*. We baited traps with (i) 1 % acetic acid +3 % ethanol and (ii) yeast-sugar solution. Twenty replicate blocks were maintained for 2 d between 22 and 24 March 2016.

Non-Target Insects

in Experiments 5 and 8 where we compared a mixture of acetic acid and ethanol with a yeast-sugar solution, we also counted non-target insects captured in traps to test the hypothesis that a mixture of acetic acid and ethanol would be more selective than a yeast-sugar solution that would contain many other fermentation volatiles as well as acetic acid and ethanol. After *P. downsi* were counted, non-target insects were categorized as three groups and counted: moths (Lepidoptera), *Polistes versicolor* (Hymenoptera: Vespidae), and other flies (calyptrate Diptera other than *P. downsi*).

Statistical Analyses

For all studies, a randomized complete block design was used with 10 ~ 30 replications. Male and female fly trap catches over 2 ~ 3 d were totaled for each replicate and analyzed with block as a random factor and different odor sources as a fixed factor using SAS Proc Mixed or Proc Glimmix (version 9.4). Fly catch data were

square-root transformed to improve normality and homoscedasticity (Zar 1984) and subjected to Proc Mixed.

However, Proc Glimmix with Poisson distribution with log link was used when transformation did not improve normality and homoscedasticity (for Experiments 6 and 7). For all experiments, the treatment means were compared using the Tukey-Kramer test ($\alpha = 0.05$) in SAS Proc Mixed or Proc Glimmix.

RESULTS

Trapping Experiments

Experiments 1 & 2:

Importance of Acetic Acid in Attraction of *P. downsi* to Yeast In Experiment 1, traps baited with yeast-sugar solution or a synthetic blend containing all 14 EAD-active compounds (14c) captured significantly greater numbers of *P. downsi* than traps baited with a six-component synthetic blend (6c) containing the six most abundant EAD active compounds including ethanol but not acetic acid ($F_{2,58} = 24.27$, $P < 0.001$; Table 1).

The numbers of flies captured in traps baited with yeast-sugar solution or the 14-component blend were not significantly different (Fig. 2a). The chemical differences between traps baited with 14c and 6c blends were eight EAD-active compounds including acetic acid (Table 1). In Experiment 2, traps baited with a 13-component blend (13c) consisting of 14c minus acetic acid, captured significantly fewer *P. downsi* than traps baited with 14c ($F_{2,18} = 13.39$, $P < 0.001$; Table 1).

In addition, numbers of *P. downsi* captured in traps baited with a 7-component blend (7c) of 6c plus acetic acid were not significantly different from numbers of *P. downsi* in traps baited with 14c, but were significantly greater than numbers of *P. downsi* in traps baited with 13c (Fig. 2b).

Experiment 3:

Importance of Ethanol in Attraction of *P. downsi* to Yeast When components other than acetic acid of the seven-component blend (7c) were removed individually and

when the resulting six different six-component blends were compared to 7c, only traps lacking ethanol (7c – EtOH; Fig. 3) captured significantly fewer *P. downsi* than traps baited with any of the other treatments, while numbers of flies captured by these other treatments were not significantly different from each other ($F_{6,78} = 5.97$, $P < 0.001$; Fig. 3).

Experiments 4 & 5:

Comparison of Acetic Acid + Ethanol and Yeast In Experiment 4, the numbers of *P. downsi* captured in traps baited with a mixture of acetic acid and ethanol (0.03 % acetic acid and 3 % ethanol as tested in Experiments 1–3) were not significantly different from numbers in traps baited with blend 7c ($F_{1,19} = 0.22$, $P = 0.645$; Fig. 4a).

However, the numbers of *P. downsi* in traps baited with the mixture of 0.03 % acetic acid and 3 % ethanol were significantly lower than numbers of *P. downsi* captured in traps baited with yeast-sugar solution (Experiment 5; $F_{1,19} = 11.48$, $P = 0.003$; Fig. 4b).

Experiments 6 & 7:

Optimization of the Amounts of Acetic Acid and Ethanol In Experiment 6, when acetic acid doses were tested in the lower range between 0.01 % and 0.3 %, traps baited with the mixture of 0.3 % acetic acid and 3 % ethanol captured the greatest numbers of *P. downsi* and significantly greater numbers of *P. downsi* than the mixture of 0.01 % acetic acid and 3 % ethanol ($F_{3,57} = 3.79$, $P = 0.015$; Fig. 5a).

As the dose-response at this acetic acid range appeared positive, a higher acetic acid dose range also was tested. At the higher range, between 0.3 % and 9 % acetic

acid, traps baited with the mixture of 9 % acetic acid and 3 % ethanol captured lower numbers of *P. downsi* and significantly fewer *P. downsi* than the mixture of 0.3 % acetic acid and 3 % ethanol or the mixture of 1 % acetic acid and 3 % ethanol ($F_{3,26} = 4.09$, $P = 0.017$; Fig. 5b).

In the ethanol dose range test, between 0.3 % and 15 %, traps baited with the mixture of 1 % acetic acid and 3 % ethanol captured the greatest numbers of *P. downsi*, although the treatment effect was not significant ($F_{3,57} = 2.01$, $P = 0.122$; Fig. 5c).

In the dose-response test for acetic acid, there was a positive relationship between acetic acid concentration and *P. downsi* trap catches within the range of 0.01 to 0.3 % with 3 % ethanol ($y = 0.6 + 3.2\times$, $r2 = 0.96$, $P = 0.044$, where y = number of *P. downsi* trapped and x = % concentration), while there was a negative relationship within the acetic acid range of 0.3 % to 9 % with 3 % ethanol ($y = 2.1–0.2\times$, $2r = 0.96$, $P = 0.037$).

We did not observe such a change with ethanol in the range between 0.3 % and 15 % with 0.03 % acetic acid ($y = 0.7 + 0.01\times$, $r2 = 0.33$, $P = 0.671$).

Experiment 8:

Comparison of Optimized Acetic Acid + Ethanol and Yeast The numbers of *P. downsi* in traps baited with the mixture of the optimized quantities of acetic acid and ethanol (1 % and 3 %, respectively) were not significantly different from the number of *P. downsi* captured in traps baited with yeast-sugar solution ($F_{1,19} = 0.09$, $P = 0.774$; Fig. 6).

Non-Target Insects

Totals of 1224 and 483 *P. versicolor*, 180 and 119 non-target moths (Lepidoptera), and 760 and 172 non-target flies were captured in Experiments 5 and 8, respectively. Numbers of Polistes wasps were 24.0-fold (Experiment 5, $F_{1,19} = 172.70$, $P < 0.001$) and 3.6-fold (Experiment 8, $F_{1,19} = 38.95$, $P < 0.001$) higher in traps baited with yeast-sugar solution compared to a mixture of acetic acid and ethanol (Table 2).

Similarly, numbers of moths were 22.9-fold (Experiment 5, $F_{1,19} = 85.44$, $P < 0.001$) and 22.8-fold (Experiment 8, $F_{1,19} = 31.62$, $P < 0.001$) higher in traps baited with yeast-sugar solution compared to a mixture of acetic acid and ethanol (Table 2).

Numbers of non-target flies captured were not significantly different in traps baited with a mixture of 0.03 % acetic acid and 3 % ethanol compared to a yeast-sugar solution (Experiment 5, $F_{1,19} = 0.00$, $P = 0.996$), while they were 74.4 % higher in traps baited with a mixture of 1 % acetic acid and 3% ethanol compared to a yeast-sugar solution (Experiment 8, $F_{1,19} = 16.04$, $P < 0.001$; Table 2).

DISCUSSION

Our results show that acetic acid and ethanol are two key volatiles involved in the attraction of *P. downsi* to actively fermenting yeast-sugar solution, and suggest strong synergy between acetic acid and ethanol. Among 10 blends of synthetic compounds tested in Experiments 1, 2, and 3, only the blends that contained both acetic acid and ethanol were attractive to *P. downsi*.

When either acetic acid or ethanol was subtracted from some of the blends, the attractiveness to *P. downsi* decreased significantly. Similar synergism has been reported in other schizophorous Diptera including muscid pests, *Muscina stabulans* and *Fannia canicularis* (Landolt et al., 2015) and drosophilids, *Drosophila suzukii* (Cha et al., 2012b, 2014a; Landolt et al., 2012), *Zaprionus indianus* (Cha et al., 2014b; Epsky et al., 2014) and is believed to be related to foraging for carbohydrates.

Although sugar is an important energy source, it is odorless, making long-distance location difficult. Thus, it has been suggested that insects may rely on volatile products of sugar metabolism produced by ubiquitous microbes to locate sugar sources (Landolt et al., 2015).

Ethanol is produced from fermentation of sugar by yeasts, and acetic acid is produced by acetic acid bacteria fermentation of ethanol, although yeasts also can produce small amounts of acetic acid when acetaldehyde is converted to acetate by aldehyde dehydrogenase (Modig et al., 2002).

While acetic acid and ethanol are two key attractants involved in *P. downsi* attraction to yeast, other EAD-active yeast fermentation volatiles may increase

attraction to the mixture of acetic acid and ethanol. For example, although the 14-component blend that contained 0.03 % acetic acid and 3 % ethanol in Experiment 1 was as attractive as yeast-sugar solution, the 2-component blend composed of only 0.03 % acetic acid and 3 % ethanol in Experiment 5 was not as attractive to *P. downsi* as yeast-sugar solution.

We improved the attractiveness of the acetic acid and ethanol mixture by optimizing the concentrations of acetic acid and ethanol, and the resulting 2-component blend of 1 % acetic acid and 3 % ethanol in Experiment 8 was as attractive as yeast-sugar solution to *P. downsi*.

At this point, it is not known whether the acetic acid concentration in the actual yeast-sugar solution was close to 0.03 % or 1 %, although the acetic acid concentration was 0.03 % in a yeast fermentation process (e.g., Erasmus et al., 2004). It is also possible that there was acetic acid produced by acetic acid bacteria, as we did not sterilize samples in both laboratory and field.

Future research is necessary to determine the concentration of acetic acid in the yeast-sugar solution used in this study. If the concentration was indeed lower than 1 %, the role of other yeast EAD-active compounds should be determined to further improve the attractiveness of acetic acid and ethanol mixture to *P. downsi*.

In the dose-response test for acetic acid, there was a positive relationship between acetic acid concentration and *P. downsi* trap catches within the range of 0.01 to 0.3 % with 3 % ethanol, while there was a negative relationship within the acetic acid

range of 0.3 to 9 % with 3 % ethanol. We did not observe such a relationship with the ethanol range between 0.3 % and 15 % with 0.03 % acetic acid.

For certain other muscid flies, a positive relationship has been reported over the acetic acid concentrations between 0.008 % and 2 % with 5 % ethanol and also ethanol concentrations from 0.2 % and 25 % with 0.5 % acetic acid (Landolt et al., 2015), suggesting that different fly species may have differences in optimal ranges of acetic acid and ethanol. It still is not clear how acetic acid and ethanol are perceived and processed in insect attraction (Cha et al., 2012b).

For many insect species, acetic acid elicits avoidance behavior, especially at high concentrations, and Ai et al. (2010) reported a highly selective olfactory neuron for detection of acids that generally is related to acid-avoiding behavior in *D. melanogaster*, although it is well known that *D. melanogaster* is attracted to acetic acid. This suggests complexity in the processing of sensory information related to acetic acid.

For example, it has been shown that egg-laying preference for and positional aversion to food containing acetic acid by *D. melanogaster* is mediated by trade-offs in two distinct sensory modalities the preference for egg-laying on acid-containing food depends on gustatory inputs, and the positional avoidance for acetic acid containing food depends on olfactory inputs (Joseph et al., 2009). Even less information is available for insect olfactory perception and processing of ethanol (Cha et al., 2012b).

Our non-target results generally provided support for the hypothesis that a mixture of acetic acid and ethanol attracts fewer non-target insects than yeast-sugar solution. For *P. versicolor* and moths, the reduction was consistent and often striking.

As predicted, traps baited with both the original acetic acid and ethanol lure (0.03 % acetic acid and 3 % ethanol) and the optimized lure (1 % acetic acid and 3 % ethanol) consistently captured significantly fewer Polistes and moths compared to the traps baited with yeast-sugar solution. All the Polistes trapped were *P. versicolor*, also an invasive pest in Galapagos (Causton et al., 2006).

Our results suggest that yeast-sugar volatiles may be a productive starting point for developing attractant lures for *P. versicolor*. Unlike Polistes and moths, however, non-target flies did not differentiate traps baited with the original acetic acid and ethanol lure and traps baited with yeast-sugar solution. This suggests that these nontarget insects also respond to acetic acid and ethanol.

Similar to *P. downsi*, the non-target flies also appeared to respond better to the optimized acetic acid and ethanol lure with significantly more non-target flies caught in traps baited with the optimized blend compared to traps baited with yeast-sugar solution.

These results indicate that in future studies it will be important to identify non-target flies by species in order to determine if there are endemic or native species that could be adversely affected by pest management based on semiochemicals. It is possible that the synthetic lure and natural bait attracted different species of nontarget flies in different proportions.

Future studies will test whether acetic acid and ethanol can be released together from a long-lasting dispenser (instead of from the drowning solution), whether mass trapping can suppress *P. downsi* infestation, how many and where traps are needed to

decrease infestation effectively, and the effect of habitat characteristics on attraction efficiency and selectivity.

This last question is especially important as previous attempts to trap *P. downsi* in the area of the critically endangered mangrove finch have generally not been successful using traps baited with papaya juice or yeast, although the rate of parasitism on the mangrove finch and others was high in the experimental area (Cunninghame et al. 2015, F. Cunninghame pers. Comm.). Vegetation structure, microclimatic factors, and competing sources of attraction may all influence semiochemical efficiency.

In conclusion, as the optimized acetic acid and ethanol lure was as attractive as actively fermenting yeast-sugar solution, we anticipate that these compounds can be used to develop a highly attractive semiochemical lure with potential for use in monitoring and mass trapping. It also is apparent that a chemically defined and simple lure tailored to attract *P. downsi* may also attract fewer non-target insects than food-based baits.

Table 1 compound name, release rate (mg/day/150 ml yeast-sugar solution), and ratio (%) of the EAD-active volatiles from the headspace of yeast-sugar solution, and the amount loaded (μl/150 ml water/trap) in different chemical blends (14c, 13c, 6c, and 7c) used in field experiments. *The concentration of acetic acid and ethanol in yeast-sugar solution is based on Banat et al. (1998) and Erasmus et al. (2004). Symbol ○ indicates whether a chemical component was included in a tested blend that was released from the trap drowning solution.

EAD-active compounds	Release rate (mg/day/150 ml)	Ratio (%)	Loading (μl/trap)	14c	13c	6c	7c
Ethyl butyrate	0.3	0.4	0.4	○	○		
1-propanol	1.3	1.4	1.6	○	○		
Isobutanol	26.6	29.1	33.2	○	○	○	○
3-methylbutyl acetate	0.9	1.0	1.1	○	○		
2-methylbutyl acetate	0.2	0.2	0.2	○	○		
3-methyl butan-1-ol	39.2	42.9	48.1	○	○	○	○
2-methyl butan-1-ol	9.2	10.1	11.3	○	○	○	○
Ethyl hexanoate	0.8	0.9	0.9	○	○		
Acetoin	3.5	3.9	3.6	○	○	○	○
2,3-butanediol	0.8	0.8	0.8	○	○		
2-phenylethyl acetate	0.3	0.3	0.3	○	○		
2-phenylethanol	8.2	8.9	8.0	○	○	○	○
Acetic acid		0.03*	45	○			○
Ethanol		3*	4500	○	○	○	○

[a]The concentration of acetic acid and ethanol in yeast-sugar solution is based on Banat et al. (1998) and Erasmus et al. (2004). Symbol ○ indicates presence of a chemical in the corresponding blend.

Table 2 Mean (±SE) numbers of non-target *Polistes versicolor*, moths (Lepidoptera), and other flies captured in traps baited with an actively fermenting yeast-sugar solution (Yeast) or a mixture of 0.03% acetic acid and 3 % ethanol (experiment 5) and traps baited with an actively fermenting yeast-sugar solution (Yeast) or a mixture of 1 % acetic acid and 3 % ethanol (experiment 8). For each non-target group, within each experiment, different letters on means indicate significant differences by Tukey–Kramer tests at $P < 0.05$. Statistical tests were based on square-root transformed data. Means from untransformed data are shown.

Treatments	*Polistes versicolor*	Moths	Other flies
Experiment 5			
Yeast	117.5 ± 11.68 a	17.2 ± 2.84 a	36.4 ± 4.27 a
0.03 % Acetic acid +3 % Ethanol	4.9 ± 0.89 b	0.75 ± 0.23 b	39.6 ± 6.85 a
Experiment 8			
Yeast	37.9 ± 5.96 a	11.4 ± 2.40 a	6.25 ± 0.87 b
1 % Acetic acid +3 % Ethanol	10.4 ± 2.22 b	0.5 ± 0.14 b	10.9 ± 1.17 a

Identification of EAD-Active Volatiles from Yeast

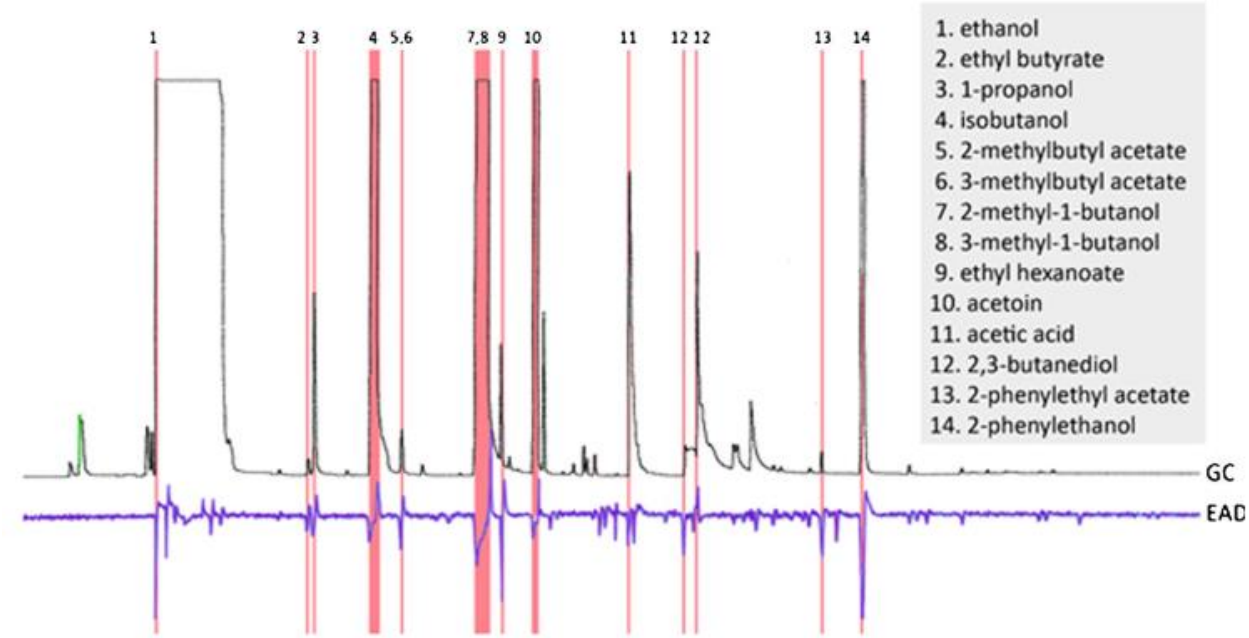


Fig. 1 Analysis of yeast-sugar fermentation headspace volatiles by gas chromatography with electroantennographic detection (GC-EAD) on polar GC column showing 14 responses from antennae of *Philornis downsi.* Compounds that consistently elicited EAD responses were identified as (1) ethanol, (2) ethyl butyrate, (3) 1-propanol, (4) isobutanol, (5) 2-methylbutyl acetate, (6) 3-methylbutyl acetate, (7) 2-methyl-1-butanol, (8) 3-methyl-1-butanol, (9) ethyl hexanoate, (10) acetoin, (11) acetic acid, (12) 2,3-butanediol, (13) 2-phenylethyl acetate, and (14) 2-phenylethanol. The release rates, relative amounts, and the amounts used in trapping experiments of the EAD-active volatiles produced by yeast are listed in Table 1. The amount of combined EAD-active volatiles released from 150 ml of yeast-sugar solution, excluding acetic acid and ethanol, was 91.3 mg/day/150 ml yeast-sugar solution.

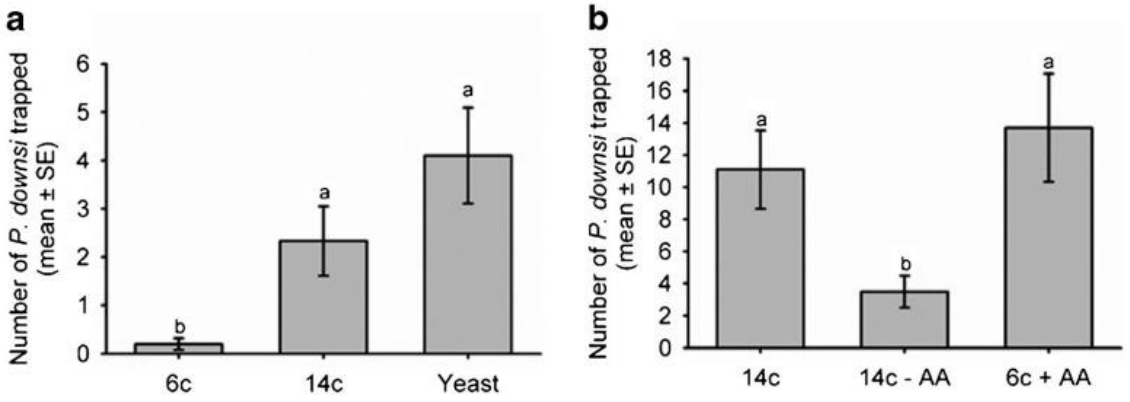


Fig. 2 Mean (± SE) numbers of *Philornis downsi* adult flies captured in (**a**) Experiment 1 with traps baited with the six-component lure (6c; Table 1), the 14-component lure (14c), or an actively fermenting yeast-sugar solution (Yeast) and (**b**) Experiment 2 with traps baited with the 14-component lure, the 13-component lure (14c minus acetic acid; 14c – AA), or the seven component lure (6c plus acetic acid; 6c + AA). For each graph, different letters on bars indicate significant differences by Tukey–Kramer tests at $P < 0.05$. Statistical tests were based on square-root transformed data. Means from untransformed data are shown. Total catches in Experiments 1 and 2 were 199 and 283 *P. downsi*, respectively.

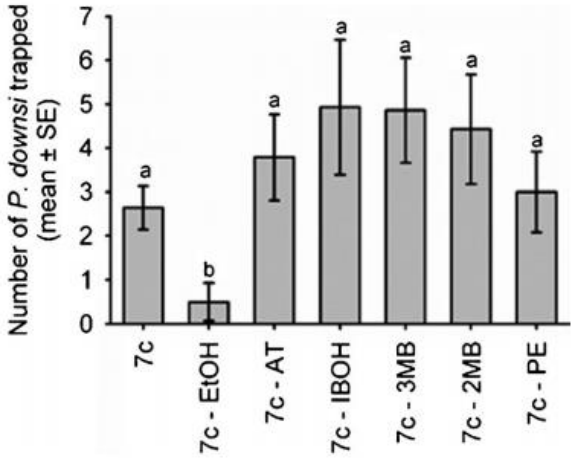


Fig. 3 Mean (± SE) numbers of *Philornis downsi* adult flies captured in Experiment 3 with traps baited with the seven-component blend (7c; Table 1), the seven-component blend minus ethanol (7c − EtOH), the seven-component blend minus acetoin (7c − AT), the seven-component blend minus isobutanol (7c − IBOH), the seven-component blend minus 3-methylbutan-1-ol (7c – 3MB), the seven-component blend minus 2-methylbutan-1-ol (7c – 2MB), or the seven-component blend minus 2-phenylethanol (7c – PE). Different letters on bars indicate significant differences by Tukey–Kramer tests at $P < 0.05$. Statistical tests were based on square-root transformed data. Means from untransformed data are shown. Total catch of *P. downsi* was 338.

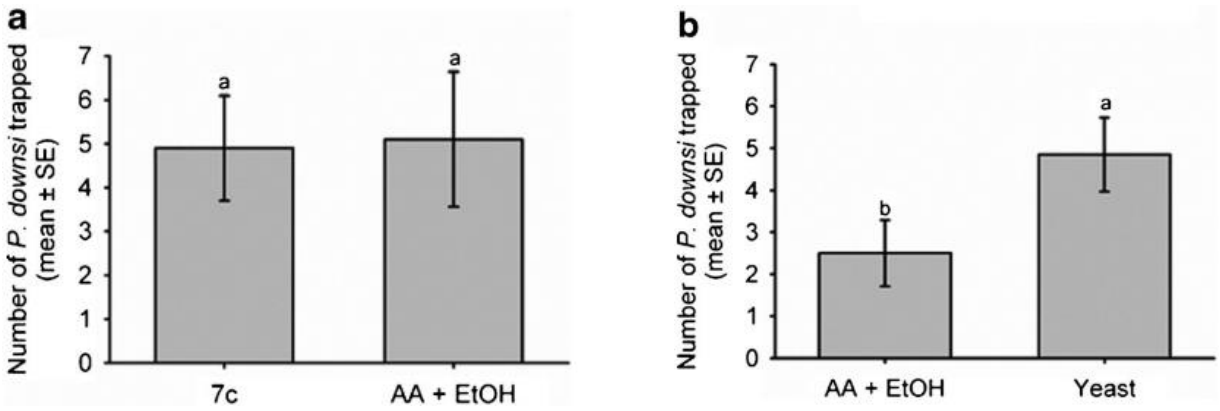


Fig. 4 Mean (± SE) numbers of *Philornis downsi* adult flies captured in (**a**) Experiment 4 with traps baited with the seven-component lure (7c; Table 1) or the mixture of 0.03 % acetic acid and 3 % ethanol (AA + EtOH) and (**b**) Experiment 5 with traps baited with the mixture of 0.03 % acetic acid and 3 % ethanol (AA + EtOH) or an actively fermenting yeast-sugar solution (Yeast). For each graph, different letters on bars indicate significant differences by Tukey–Kramer tests at $P < 0.05$. Statistical tests were based on square-root transformed data. Means from untransformed data are shown. Total catches *P. downsi* were 200 and 147 in Experiments 4 and 5, respectively.

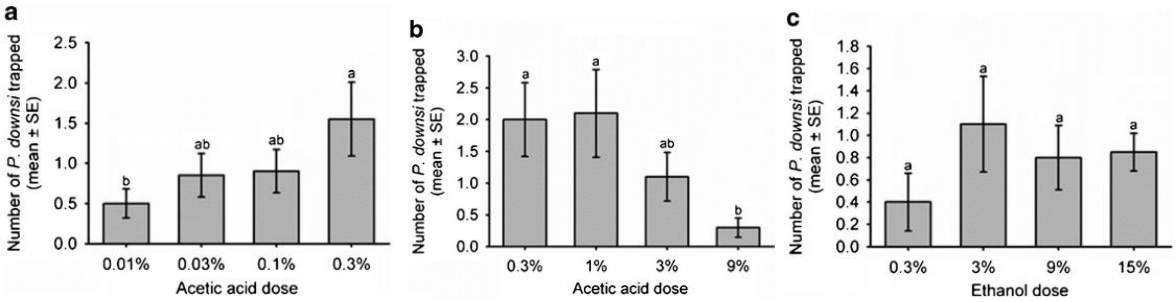


Fig. 5 Mean (± SE) numbers of *Philornis downsi* adult flies captured in traps baited with different concentration (indicated as % in 150 ml trap drowning solution) of acetic acid with 3 % ethanol in Experiment 6 (**a, b**) and different concentration (%) of ethanol with 1 % acetic acid in Experiment 7 (**c)**. For each graph, different letters on bars indicate significant differences by Tukey-Kramer tests at $P < 0.05$. Total catches of *P. downsi* in Experiments 6 and 7 were 99 (76 flies for 0.01 ~ 0.3 % acetic acid and 23 for 0.3 ~ 9 % acetic acid), respectively.

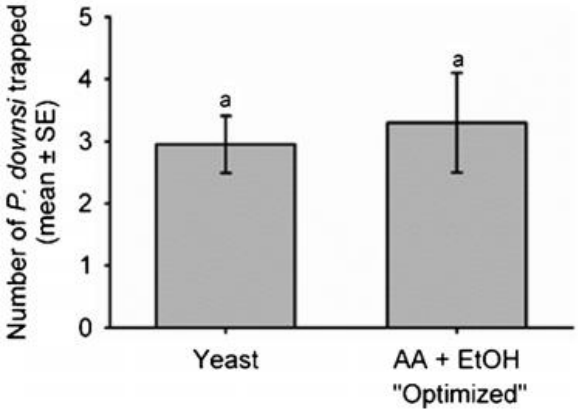


Fig. 6 Mean (± SE) numbers of *Philornis downsi* adult flies captured in Experiment 8 with traps baited with an actively fermenting yeast-sugar solution (Yeast) or the optimized mixture of 1 % acetic acid and 3 % ethanol (AA + EtOH “Optimized”). There was no significant difference between treatments (Tukey–Kramer tests at $P<0.05$). Statistical tests were based on square-root transformed data. Means from untransformed data are shown. Total catch of *P. downsi* was 125.

CHAPTER III

Trapping of *Philornis downsi* (Diptera: Muscidae) with ethanol and acetic acid with controlled release dispensers

To be submitted to J Chem Ecol.

INTRODUCTION

Philornis downsi (Diptera: Muscidae) is a hematophagous fly that primarily parasitizes passeriform birds and near relatives. Only the larval stages feed on the blood of nestlings. In the first developmental stage, *P. downsi* larvae feed within the nasal cavities of nestlings; in later developmental stages the larvae feed externally at night and are found in the chicks' thighs, abdomen and below the wings (Fessl et al., 2006).

This parasitic fly is now present on 15 of the 17 main islands of the Galapagos archipelago, and has been classified as one of the greatest threats to the avian diversity of the archipelago (Fessl et al., 2018).

Over the last decade, many studies have been carried out to determine the negative impacts of *P. downsi* on its avian hosts. One of these studies indicated that in three species of finches nestling blood loss from parasitism could be as high as 18 to 55%; blood volume loss greater than 25% can be lethal to the chicks (Fessl et al., 2006).

Other studies confirmed that blood loss from parasitism resulted in low hemoglobin levels and decreased growth of chicks (Dudaniec, 2006). Low hemoglobin

levels (anemia) during the high metabolic demand phase of nestling growth logically lead to increased mortality (Dudaniec, 2008). This in turn results in high levels of nest abandonment which could lead to local extinctions of Darwin finch populations (Fessl et al., 2018).

When nestlings survive to maturity, the physical damage and deformation from parasitism during early development could reduce mating opportunities as adults, thus adding to the potential for population decline and local extirpation (Grant et al., 2005; Galligan & Kleindorfer, 2009).

In insects, the search for mates, food sources and oviposition sites often involves attraction to semiochemicals such as pheromones, kairomones, and food odors (Landolt et al., 2012). Food odors may affect fly behavior, either as a stimulus for oviposition or as a trigger to initiate courtship and subsequent mating (Becher et al. 2010; Jallon 1984; Joseph et al., 2009).

For example, Landolt et al. (2012) showed that large numbers of *Drosophila susukii* can be captured in traps baited with acetic acid and ethanol odors and determined that this mixture was more efficient than a vinegar solution.

In order to mitigate the negative effects of *P. downsi* on Galapagos land birds, several control and monitoring strategies are under development. These control strategies will require identification of chemical attractants such as food odors (Cha et al., 2016). Adult *P. downsi* differ from the larval stage in that they mainly feed on decomposing fruits (Fessl et al., 2018).

Potential attractive food odors are therefore likely to include volatile compounds produced by microorganisms such as bacteria and fungi during fermentation (Tait et al.,

2014). Consistent with this hypothesis, Cha et al. (2016) showed that volatiles such as ethanol and acetic acid produced by baker's yeast (*Saccharomyces cerevisiae*) during the fermentation of a sugar solution were attractive to *P. downsi*.

The attractiveness of compounds produced by yeast fermentation has been evaluated previously in flies of the family Drosophilidae. A study conducted by Kleiber et al. (2014) suggests that compounds related to fermentation products such as acetic acid, ethanol, ethyl acetate and 2-phenethyl alcohol were effective in attracting *D. suzukii*.

However, combining the compounds did not significantly increase capture rates. Some lures made with isolated compounds from yeasts may attract moths and flies of both sexes which makes these baits useful for sampling, monitoring, or controlling pests in agroecosystems (Landolt, 1993; Landolt & Alfaro, 2001).

Key to the success of any compound that has potential as a lure is the evaporation rate and longevity in the field. Methods have been developed to predict the rates and times of evaporation of chemical mixtures that are potential lures (Ranz & Marshall, 1952; Spedding et al.,1993).

However, when the mixture includes two or more compounds that evaporate at different rates, measurement becomes more complicated. Although predictions of time-varying evaporation rates can be made for chemical mixtures, these estimates require empirical verification prior to application (Smith, 2001).

The GC-EAD assays of Cha et al. (2016) indicated that *P. downsi* antennae can detect 14 compounds produced during yeast fermentation. These compounds may be attractants that could be effective in monitoring and control of *P. downsi*. Cha et al.

(2016) systematically evaluated combinations of these compounds in the laboratory and in the field for attraction of *P. downsi*.

Field trials indicated that the most attractive compounds for *P. downsi* adults were ethanol and acetic acid in a ratio of 0.03% acetic acid (AA) and 3% ethanol (EtOH). This combination was equally attractive as the full 14-component mixture and was as attractive as a yeast-sugar solution. In addition, these trials indicated that the simplified two component chemical lure attracted fewer non-target insects than food-based baits (Cha et al., 2016).

A potential problem with the simplified lure, however, is that the attraction of the mixture of these two compounds is fleeting due to their evaporation and rapid depletion. The solution must be replaced frequently in order to maintain attractiveness of the trap. Moreover, it is difficult to determine the optimum rates of evaporation of the attractants when the solution is used in McPhail traps.

Thus, a quantitative analysis of the rates of ethanol and acetic acid evaporation from the water solution in the trap is needed, as well as methods to manipulate the evaporation rates of these compounds. Ideally, a controlled release dispenser system would be deployed that would allow optimum chemical release of the blend to be used in McPhail traps making the EtOH and acetic acid blend attractive for a longer period of time, e.g. weeks rather than days.

This study was designed to address these concerns by:

1) Determining the evaporation rates of ethanol and acetic acid individually and in aqueous solutions.

2) Evaluating release rates of the ethanol and acetic acid mixture from experimental dispensers in the field.

3) Determining the attraction of different ethanol and acetic acid dispensers for increased efficiency of *P. downsi* capture.

MATERIALS AND METHODS

Laboratory investigation of ethanol and acetic acid volatilization rates

Chemicals:

For one liter of the lure solution, 965.4 ml of water was mixed with 31.6 ml of a 3% solution of ethanol and 3 ml of a 0.3% solution of glacial acetic acid. Tap water was used in the lure solution in the field in Galapagos.

The water was assumed to have a mass per mole of 18.02 g mol−1 at 2O°C. Stock ethanol (Pharmco; CAS No. 64-47-5) with a mass per mole of 46.07 g mol−1 and purity ≥ 99.5% and glacial acetic acid (Pharmco; CAS No. 64-19-7) with a mass per mole of 60.05 g mol-1 and purity ≥ 99.4% (wt) were used as attractants in solution.

Characteristics of ethanol and acetic acid

Ethanol or ethyl alcohol (CH3-CH2-OH) is a two carbon alcohol concentrated by distillation of dilute solutions. Commercial preparations contain 95% by volume of ethanol and 5% of water. Commercial ethanol has a relative density of 0.789 at 20 °C. Its diffusion coefficient (*D*) in air at standard temperature and pressure (25°C, 760 mm) is 11.81 x 10^{-2} cm^2s^{-1}(Lugg, 1968).

Glacial acetic acid, also called ethanoic acid or methylene carboxylic acid (CH_3-COOH), is a two carbon organic acid that commonly occurs in the form of an acetate ion (CH_3-COO^-) in water. The carboxyl group (-COOH) confers acidic properties to the molecule. This is the acid responsible for the tart taste and smell of vinegar. Acetic acid

has a relative density of 1.049 g cm-3 at 20°C. Its diffusion coefficient in air at standard temperature and pressure (25°C, 760 mm) is 12.35 x 10^2 cm^2s^{-1} (Lugg, 1968).

Note that the higher the diffusivity of one substance with respect to another in solution, the faster the diffusion rate will be for both of the substances. Ethanol and acetic acid are very close in diffusivity in air, but the diffusion coefficient of water in air is 0.282 cm^2s^-1, much lower than the two carbon molecules.

Furthermore, the diffusion coefficient for ethanol in water at 25°C is 8.4x10^{-6} cm^2 s^{-1} while the *D* for acetic acid in water is 12.1 x10^{-6} cm^2 s^{-1}. Thus, ethanol and acetic acid should both diffuse rapidly into water, but the evaporative behavior of the resulting aqueous solution containing both molecules will depend upon the water.

Thus, the complex nature of diffusivity of a solution containing multiple chemical compounds is best understood through empirical measurements, such as presented here.

Experiment 1: Evaporation in a confined space.

This experiment measured the rate of evaporation of a solution of ethanol and acetic acid, with or without water, contained in a vessel with a reduced aperture. Volumetric flasks (10 ml; Pyrex®) with a 5 mm opening at the top were used to allow vapor to escape. One of seven treatment solutions (10 ml each) was placed in each volumetric flask:

Treatment 1 (T1) 3% EtOH + 0.3% AA in water

Treatment 2 (T2) 97% EtOH + 3% AA

Treatment 3 (T3) 3% EtOH in water

Treatment 4 (T4) 0.3% AA in water

Treatment 5 (T5) 99.5% EtOH

Treatment 6 (T6) 99.4% AA

Control (10 ml water)

Volume and mass (laboratory balance Model AR3130 Ohaus Corp., Pine Brook, NJ) were measured every hour for six hours with six replications.

Experiment 2: Evaporation in open space.

In this experiment we measured the rate of evaporation of ethanol and acetic acid, with and without water, in glass beakers (50 ml; Corning Pyrex® low-form) with a 5 cm opening to allow a larger surface area for evaporation of test solutions (Figure 1b). Seven beakers were used each containing 50 ml of one of the following:

Treatment 1 (T1) 3% EtOH + 0.3% AA in water

Treatment 2 (T2) 97% EtOH + 3% AA

Treatment 3 (T3) 3% EtOH in water

Treatment 4 (T4) 0.3% AA in water

Treatment 5 (T5) 99.5% EtOH

Treatment 6 (T6) 99.4% AA

Control (50 ml water)

Mass and volume were measured every 60 minutes for 6 hours with six replicates.

Experiment 3: Evaporation in McPhail traps.

This experiment provide an approximation of the evaporation rates of aqueous solutions of ethanol and acetic acid in McPhail traps, but under a laboratory setting. The dimensions of the McPhail traps used for this part of the study were 22 cm h ×17 cm dia. and the diameter of the funnel was 9 cm. One of three aqueous solutions (150 ml) was placed in each McPhail trap:

Treatment 1 (T1) 3 % EtOH

Treatment 2 (T2) 0.3 % AA

Treatment 3 (T3) 3 % EtOH + 0.3 % AA

Mass and volume were measured every 24 hours for five days.

Experiment 4: External reservoir.

In this experiment, a medical intravenous drip system Exel I.V. Pic 1a (Medical and Veterinary supplies, Mettawa, IL, USA) was used to continuously add 3% of EtOH + 0.3% of acetic acid solution to a volumetric micro flask (1 ml; Pyrex®) with a 3-mm opening at the top to allow vapor to disperse.

The drip rate was one drop per 15 minutes until 1 ml was reached in the flask. After filling the flask, the contents of the flask and the bag were weighed to determine the amount of solution that evaporated during the filling of the flask. Mass and volume were measures and were recorded every 60 minutes for 6 hours with two replicates.

The initial masses and volumes of the liquids from the four experiments were recorded. The containers of liquid were placed inside a stainless steel laboratory oven

with adjustable temperature control to 550°F / 287°C max (Global Equipment Company Inc., Port Washington, New York, USA); oven temperature was held steady at 32°C. A polyethylene tube was used to transfer bench vacuum (airflow of 500 ml/min) to the oven to remove volatiles.

Field trapping experiments

Three methods of releasing AA and EtOH into McPhail traps were tested. First, the attractant solutions were placed in carbon-impregnated polyethylene bags (CIPB; Alpha Scents, Inc., West Linn, OR, USA), which allow controlled penetration by ethanol (Figure 2a). Second, the controlled release of the EtOH and AA solution from reservoir bags was achieved using an IV drip system (external reservoir) (Figure 2b).

Lastly, to retard any decomposition of captured insects and potentially increase trap captures of *P. downsi*, boric acid or propylene glycol was added to the trap drowning solution. Traps were installed at El Barranco (0°44'25"S, 90°18'25"W) in the arid lowlands of Santa Cruz Island.

Experiment 5: Dose-range assay of CIPBs.

The following 150 ml of aqueous solutions were placed in CIPBs (7.5 x 12 cm), which were then placed in McPhail traps:

Treatment 1 (T1) 0.03% AA + 0.3% EtOH + 99.67% water;

Treatment 2 (T2) 0.1% AA + 1% EtOH + 98.9% water;

Treatment 3 (T3) 0.3% AA + 3% EtOH + 96.7% water;

Treatment 4 (T4) 1% AA + 10% EtOH + 89% water.

A drowning solution (150 ml) consisting of water and an odorless detergent to break the surface tension of the water was placed in the base of the traps. A randomized complete block design with 10 blocks was used. The traps were placed in the field from April 6 to 10, 2017. *P. downsi* were removed each day, and the traps were moved one position within the block to reduce position effects. Trapping was performed with 15 replicates (N=45).

Experiment 6: Effects of propylene glycol and high release rates.

In order to improve the attraction of the drowning solution, propylene glycol (PG) was evaluated as a preservative. Due to its antifungal and antimicrobial properties, this product might prevent odors produced by decomposing insects within the trap. However, propylene glycol might also change the chemical attractive properties of the EtOH and AA solution.

Propylene glycol acts as a surfactant and in the presence of EtOH, can strongly bind water (Szumała, 2015), possibly altering evaporation rates and the effectiveness of the lure solution. Three experimental treatments were therefore used to empirically test the effects of adding propylene glycol to the lure solution as a potential decomposition retardant:

Treatment 1 (T1) 150 ml drowning solution of 1% AA + 3% EtOH + 96% water

Treatment 2 (T2) Blend of 1% AA + 10% EtOH + 89% water in CIPBs plus drowning solution of 150 ml water

Treatment 3 (T3) 150 ml drowning solution of 1% AA + 10% EtOH + 20% propylene glycol + 69% water

In all treatments the drowning solution was placed in the base of the McPhail traps along with a drop of odorless detergent. Traps were set out in a randomized block design. The traps were shifted one position within the block each day and the flies were removed. There were 10 spatial blocks and four temporal (daily) replicates (N=40) from April 6 to 16, 2017.

Effects of boric acid and controlled release.

Similar to the experiment described above, the main objective of the next set of experiments was to minimize the decomposition of specimens trapped during field experiments and the associated repellent effect of protein decomposition volatiles by the addition of boric acid to the drowning solution.

Moreover, evaporated compounds in the McPhail traps were replaced with new liquid delivered by an IV drip system (external reservoir) (Fig 1a). The effect of boric acid and the controlled addition of EtOH and AA from an external reservoir were evaluated in two experiments.

Experiment 7: Effects of boric acid and an external reservoir.

The first part of the experiment to evaluate the effectiveness of boric acid (BA) as a decomposition retardant in McPhail traps included the following treatments:

Treatment 1 (T1) 150 ml drowning solution of 0.3% AA + 3% EtOH + 96.7 % water .

Treatment 2 (T2) 150 ml drowning solution of 0.3% AA + 3% EtOH + 1% BA + 95.7% water.

Treatment 3 (T3) 150 ml drowning solution (DS) of 0.3% AA + 3% EtOH + 96.7 % water + external reservoir with 250 ml of DS.

In all treatments the drowning solution was placed in the base of the McPhail traps along with a drop of odorless detergent. Fifteen spatial replicates were used in the same experimental design as described above and the traps were installed in the field from April 28 to May 3, 2017.

Experiment 8: Effects of boric acid combined with an external reservoir.

In the second experiment, two treatments were tested:

Treatment 1 (T1) 150 ml drowning solution (DS) of 0.3% AA + 3% EtOH + 1% BA 96.7 % water + 250 ml DS with 1% of BA in a drip system (external reservoir).

Treatment 2 (T2) 150 ml drowning solution of 0.3% AA + 3 % EtOH + 1% BA + 96.7 % water.

In all treatments the drowning solution was placed in the base of the McPhail traps along with a drop of odorless detergent. Twenty spatial replicates were completed with the same experimental design as above and the traps were installed from May 5 to 9, 2017.

Statistical Analyses.

Laboratory assay data were analyzed using two-factor ANOVA with replication ($\alpha = 0.05$). Field trapping data were square root transformed to achieve normality and homoscedasticity. However, the Generalized Linear Model for Poisson distribution with log link was used when transformation did not achieve normality and homoscedasticity.

For both laboratory and field experiments, the treatment means were compared using the Tukey-Kramer test ($\alpha = 0.05$). The analyses were carried out using the statistical package R version 3.1.3 (R Core Team 2013) and R-Commander package (Fox & Bouchet-Valat, 2018).

RESULTS

Experiment 1: Evaporation in confined space.

The overall treatment effect on mass loss was significant ($F_{6,42} = 4.577$, $P = 0.001$). Tukey-Kramer tests showed statistically significant differences in mass loss between treatment two (T2), control and treatments (T3 and T6), while the volume loss registered differences between T1 and T6.

On a volume basis, there was also a significant treatment effect ($F_{6,42} = 3.173$, $P = 0.011$). Significant differences in the decrease in volume occurred between T1 and T6. Thus, 97% EtOH + 3% AA proportionally lost more mass, while the 3% EtOH + 0.3% AA in water solution lost more volume (Figure 3) (Table 1).

Experiment 2: Evaporation in open space.

Assays in 50 ml glass beakers showed patterns similar to those in Experiment 1 in the rates of mass and volume loss (Figure 3). The overall treatment effect on mass loss was significant ($F_{6,42} = 9.811$, $P < 0.001$).

Assays of open space showed statistically significant differences in mass loss between treatments T2 and T5 contrasted with control and treatments T1, T3, T4, and T6, while the volume loss register differences between treatments T2 and T5 contrasted with control and treatments T1, T3 and T4 (Tukey-Kramer tests) (Table 1).

The decrease in volume also showed a significant treatment effect ($F_{6,42} = 10.898$, $P < 0.001$). Significant differences occurred between T1 and T6. The solutions

containing higher proportions of EtOH lost proportionally more in both mass and volume.

Experiment 3: Evaporation in McPhail Traps.

Evaporation of the three solutions (0.03 % acetic acid and 3 % ETOH in water [T1 Drowning solution], 3 % EtOH in water [T2], and 0.03 % acetic acid in water [T3]) from McPhail traps were not significantly different in either the rate of mass loss ($F_{2,17}$ = 0.029, P = 0.972) or volume loss ($F_{2,17}$ = 1.210, P = 1.000 (Figure 5). Different letters next to mean values indicate significant differences in Tukey–Kramer tests at $P < 0.05$.

Experiment 4: External reservoir.

A solution (3% EtOH + 0.3% AA) dripping into a 1 ml volumetric flask filled in approximately 25 hours with an average rate of 1 drop every 15 minutes. The rate of evaporation was greater before the drop fell into the flask, that is, the solution evaporated from the surface of the drop while it was hanging from the end of the I.V. system. After 24 hours, this drip assay indicated that the amount of solution (97% EtOH + 3% AA) evaporated in the volumetric flask (1 ml) was 0.132 ml.

Experiment 5: Dose-range assay of CIPBs.

A total of 24 *P. downsi* (15 females and 9 males) were caught in the traps using CIPBs lures for the release of all concentrations evaluated of EtOH and AA. The generalized linear model showed no significant differences between four treatments and

total flies captured (X^2=2.5666, df=3, P =0.464), treatments and female flies (X^2=3.896, df=3, P =0.273), also treatments and male flies (X^2=2.133, df=3, P =0.545) (Table 2).

Experiment 6: Effects of propylene glycol and high release rates.

A total of 152 *P. downsi* (66 males and 86 females) were captured in experiment 6. The generalized linear model detected significant differences among the three treatments (Poisson regression, X^2=259.449, df=3, P< 0.0001).

The treatment using a CIPB dispenser as the delivery mechanism for 1% AA and 10% Ethanol (T2) and the treatment with propylene glycol in the drowning solution in the bottom of the McPhail trap with 1% AA and 10% Ethanol (T3) captured significantly fewer flies compared to the treatment with 150 ml drowning solution made up of 1% AA + 3% EtOH + 94% water with a drop of detergent (T1) (Tukey-Kramer tests, α=0.05; Figure 6).

Effects of boric acid and external reservoir

Experiment 7: Effects of boric acid and an external reservoir.

In this experiment, a total of 71 *P. downsi* were captured (36 males and 35 females). The highest number of flies were caught in the treatment with continuous replacement of the drowning solution through the IV drip system (external reservoir) (Table 3; Poisson regression, X^2=11.2207, df=3, P= 0.01059).

There was no significant effect of using boric acid. There was no significant effect when the sexes were analyzed separately (Sex: X^2=0.0206, df=1, P=0.88576; Treatment: sex: X^2=0.2776, df=2, P=0.87042) (Table 3).

Experiment 8: Effects of boric acid combined with an external reservoir.

A total of 541 *P. downsi* were captured (240 males and 301 females) in this experiment. The treatment without the IV drip system captured more *P. downsi* than the treatment with the IV drip system. The generalized linear model showed significant negative deviations between the treatments in the Poisson distribution (X^2=60.816, df=2, P< 0.0001) (Table 4).

DISCUSSION

These results indicate that the independent evaporation rates of ethanol and acetic acid are different from each other, yet are markedly constant over time. Acetic acid evaporation is generally negligible. Ethanol, however, evaporates faster than either water or acetic acid due to its high volatility and vapor pressure. When ethanol was added to water, the solution evaporated faster than the other solutions.

The 99.4% acetic acid in treatment 6 of Experiments 1 and 2 lost less mass and volume while maintaining its concentration compared to 99.5% EtOH. This effect was also reported by Duffey et al. (2013). The concentration of acetic acid in the aqueous solution did not change sufficiently on the time scale of Experiment 1 to affect the loss of mass and volume.

Among the containers used in these experiments (semi-closed and open), the semi-closed system was more effective at releasing smaller quantities of solutions and maintaining the original concentration at higher temperature (>32°C) and a greater air flow rate (>500 ml/min). Semi-enclosed containers appear to be useful in the laboratory (Romero and Cantero 1998) but not in the field unless the semiochemicals being released are at higher concentrations than 3% EtOH + 0.3% AA to begin with.

The effects of the size of the vessel opening and the surface of the vessel material had a significant effect on the rate of evaporation of the contained solution. O'Hare et al. (1993), Spedding et al. (1993) and Innocenzi et al. (2008) suggested that smooth surfaces may promote increased rates of evaporation.

The volume of the liquid inside the containers is also important, because when approaching the lower limit of the vessel, evaporation accelerates drastically, as was

observed in Experiments 3 and 4 with McPhail traps where this effect was observed on the fourth day.

Although Smith (2001), concluded that temperature is the main factor influencing the evaporation rates of liquids, other factors such as air flow and ambient humidity also influence evaporation rates substantially. The effect of airflow was evidenced in Experiments 3 and 4 with McPhail traps because the upper part of the container would have prevented airflow and slowed the rate of evaporation.

This is supported by a comparison of the mass of evaporated solution in the McPhail traps compared to that of the open 50-ml glass beakers that were exposed to the same airflow and temperature. In the beakers the evaporation rates were higher even though the surface area was lower. In this sense, to ensure the longevity of synthetic lures and their controlled release from a suitable dispenser, the solution should be more stable in terms of its attractiveness over time.

To extend the effective lifetime of attraction of EtOH and AA in the field, I (1) added a greater volume of solution of 3% EtOH + 0.3% of AA + water to the traps, and (2) slowed the release rate using carbon-impregnated polyethylene bags inside the McPhail traps (Experiment 5).

The solutions in the CIPBs lasted longer and probably maintained more stable concentrations, although this was not measured in the laboratory. Unfortunately the slow release of the compounds from the CIPBs was unable to attract *P. downsi* effectively.

Similarly, Landolt et al. (2012, 2015) found that higher release rates of EtOH and AA in vials with larger diameter holes attracted greater numbers of the false stable fly

and the little house fly (Diptera, Muscidae). The results of Experiment 6 in this study, showed that the placement of EtOH and AA in the drowning solution was significantly more attractive than the slower release rate from the CIPBs.

Interestingly, the addition of 20% propylene glycol to the drowning solution also significantly reduced trap catches. It is unclear whether this was due to the surfactant effect of propylene glycol altering the release rates of EtOH and AA, or possibly its ability to bind water thus changing the original odor of the lure.

The I.V. devices (external reservoirs) used to supply extra EtOH and AA solution to the traps to increase the time that the solution was attractive to *P. downsi* only marginally improved trap catches in Experiment 7 compared to when the chemicals attractants were placed in the drowning solution with and without boric acid (BA).

However, experiment 8 showed that the I.V dispenser was not more attractive than direct delivery of the lure solution into the drowning solution of the McPhail trap. This is likely due to the fact that when the lure solution is delivered rapidly to the drowning solution (no I.V. present), the increased surface area of solution releases more of the attractant molecules at one time creating in a stronger stimulus.

Furthermore, the addition of BA to the drowning solution resulted in the capture of more flies than the treatment without BA, as the insects that drowned in the solution no longer decomposed and masked the odor of the EtOH-AA mixture or had a repellent effect on adult *P. downsi*. Increased trap catches in traps with EtOH and AA and added BA was also documented by Landolt & Alfaro (2001).

The evaporation rates measured in this study can serve as a basis for estimating the effective lifetime of a solution in the field. To do this with the aqueous solution of

ethanol (3%) and acetic acid (0.3%).requires an I.V. drip feed into the McPhail traps of least 22 ml/day.

However, because the evaporation rate of EtOH is 1.8 times higher than that of AA, it should be possible to load the I.V. drip bags with a solution that has a correspondingly higher concentration of EtOH than AA.

In addition, by reducing the amount of water (and increasing the concentrations and amounts of EtOH and AA) and slowing the drip rate, it should be possible to optimize the replacement rates of EtOH and AA so that the drowning solution remains at constant concentrations of 3% EtOH and 0.3% AA, i.e., the optimum concentration determined in Chapter 2.

The results of my field experiments indicate that it is indeed possible to improve the catches of *P. downsi* for a longer period of time. This requires the addition of boric acid to the drowning solution or an external reservoir (I.V. drip system), or both. These two alternatives not only proved to be effective in capturing more flies, but they also maintained the attraction of the bait for more than five days.

Additionally, increasing the content of the trap from 150 ml to 250 ml and adding BA may be a viable alternative to the use of additional liquid with the I.V. system. However, this result is not conclusive, since catching more flies may also be due to an increase in the population of flies near the traps.

Table 1. Mean (±SE) rates of mass (g/h) and volume (ml/h) loss measured in experiments 1 and 2 under controlled conditions, assays including control (water) and six treatments in 10 ml volumetric flasks (confined space) and 50 ml glass beakers (open space).

Codes	Concentrations	Experiment 1 (10ml)		Experiment 2 (50ml)	
		Δ Mass	Δ Volume	Δ Mass	Δ Volume
Control	**water**	0.05 ± 0.06c	0.06 ± 0.01**a**	0.83 ± 0.16**a**	0.48 ± 0.10**a**
T1	**3% EtOH + 0.3% AA in water**	0.11 ± 0.14a	0.14 ± 0.02**b**	0.81 ± 0.14**a**	0.48 ± 0.09**a**
T2	**97% EtOH + 3% AA**	0.16 ± 0.12b	0.12 ± 0.03**a**	1.59 ± 0.27**bc**	2.45 ± 0.44**bc**
T3	**3% EtOH in water**	0.05 ± 0.10a	0.10 ± 0.02**a**	1.09 ± 0.20**a**	0.89 ± 0.16**a**
T4	**0.3% AA in water**	0.10 ± 0.10a	0.10 ± 0.02**a**	0.90 ± 0.15**a**	0.70 ± 0.13**a**
T5	**99.5% EtOH**	0.12 ± 0.06a	0.06 ± 0.01**a**	1.71 ± 0.28**c**	2.89 ± 0.53**a**
T6	**99.4% AA**	0.07 ± 0.06c	0.06 ± 0.01**c**	1.04 ± 0.18**a**	1.62 ± 0.29**b**

Table 2. Mean (±SE) numbers of *P. downsi* captured in experiment 5 in McPhail traps baited with four concentrations of ethanol (EtOH) and acetic acid (AA) in CIPBs.

Codes	Concentrations	Mean *P. downsi* captured	Sex	
			Female	Male
T1	**0.3% EtOH + 0.03% AA**	0.4 ± 0.03	0.4 ± 0.4	0.00 ± 0.00
T2	**1% EtOH + 0.1% AA**	0.16 ± 0.07	0.8 ± 0.37	0.8 ± 0.37
T3	**3% EtOH + 0.3% AA**	0.10 ± 0.06	0.6 ± 0.6	0.4 ± 0.4
T4	**10% EtOH + 1% AA**	0.18 ± 0.06	1.2 ± 0.58	0.6 ± 0.4

Table 3. Mean (±SE) numbers of *P. downsi* captured in experiment 7 in McPhail traps baited with 150 ml drowning solution of (EtOH 3% + AA 0.3%) with or without 1% BA or an additional 250 ml solution in an I.V drip bag.

Codes	Concentrations	Mean *P. downsi* captured	Sex	
			Female	Male
T1	**3% EtOH + 0.3% AA**	0.99 ± 0.51	0.66 ± 0.21	0.66 ± 0.40 **NS**
T2	**3% EtOH + 0.3% AA + BA**	0.99 ± 0.38	0.53 ± 0.19	0.73 ± 0.28 **NS**
T3	**3% EtOH + 0.3% AA (I.V.)**	1.56 ± 0.47	1.13 ± 0.32	1.00 ± 0.31 **NS**

Table 4. Mean (±SE) numbers of *P. downsi* captured in experiment 8 in McPhail traps baited with 150 ml drowning solution of EtOH 3% + AA 0.3% and BA (1%) in water with or without an external reservoir (I.V.) with an additional 250 ml drowning solution (IV).

Codes	Concentrations	Mean *P. downsi* captured	Sex	
			Female	Male
T1	**3% EtOH + 0.3% AA + BA +I.V.**	7.75 ± 1.55	6.70 ± 1.13	4.40 ± 0.99 ***
T2	**3% EtOH + 0.3% AA + BA**	11.78 ± 3.52	8.35 ± 2.24	7.60 ± 2.40

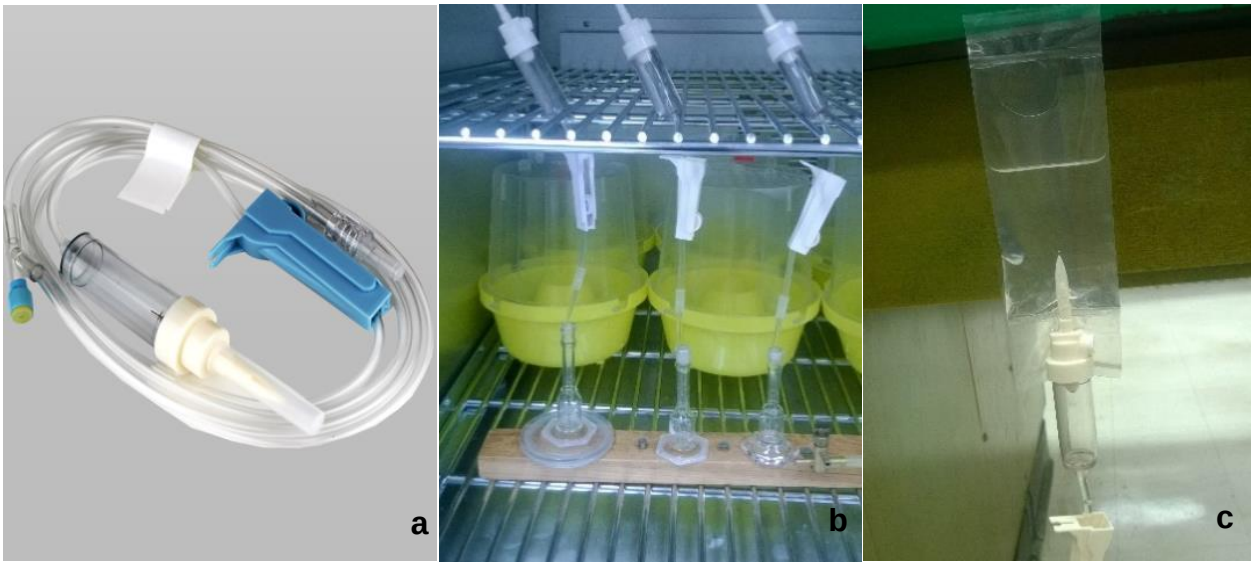


Figure 1. Intravenous drip system (IV) used in experiment 4: (a) tubing, (b) tubing and volumetric flasks, (c) I.V. attached to plastic reservoir bag.

Figure 2. McPhail traps used in assays to extend the time of attraction of the solution with ethanol and acetic acid. a) CIPB inside the trap, b) IV drip system outside of the trap.

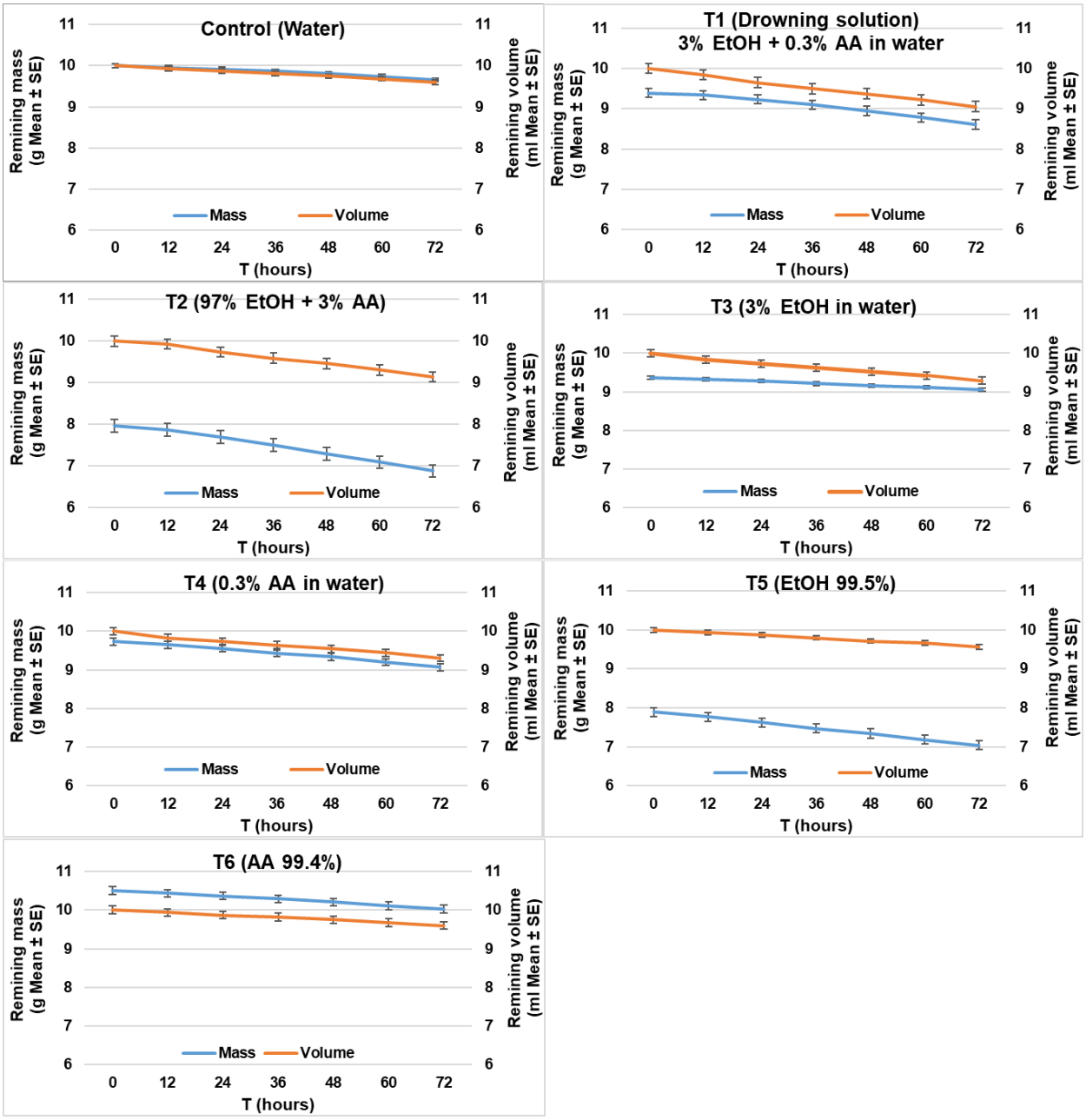


Figure 3. Loss of solution by volume and mass from 10 ml flasks in Experiment 1: Evaporation in confined space. Blue lines represent the loss of mass and red lines represent the loss of volume. Error bars represent standard error.

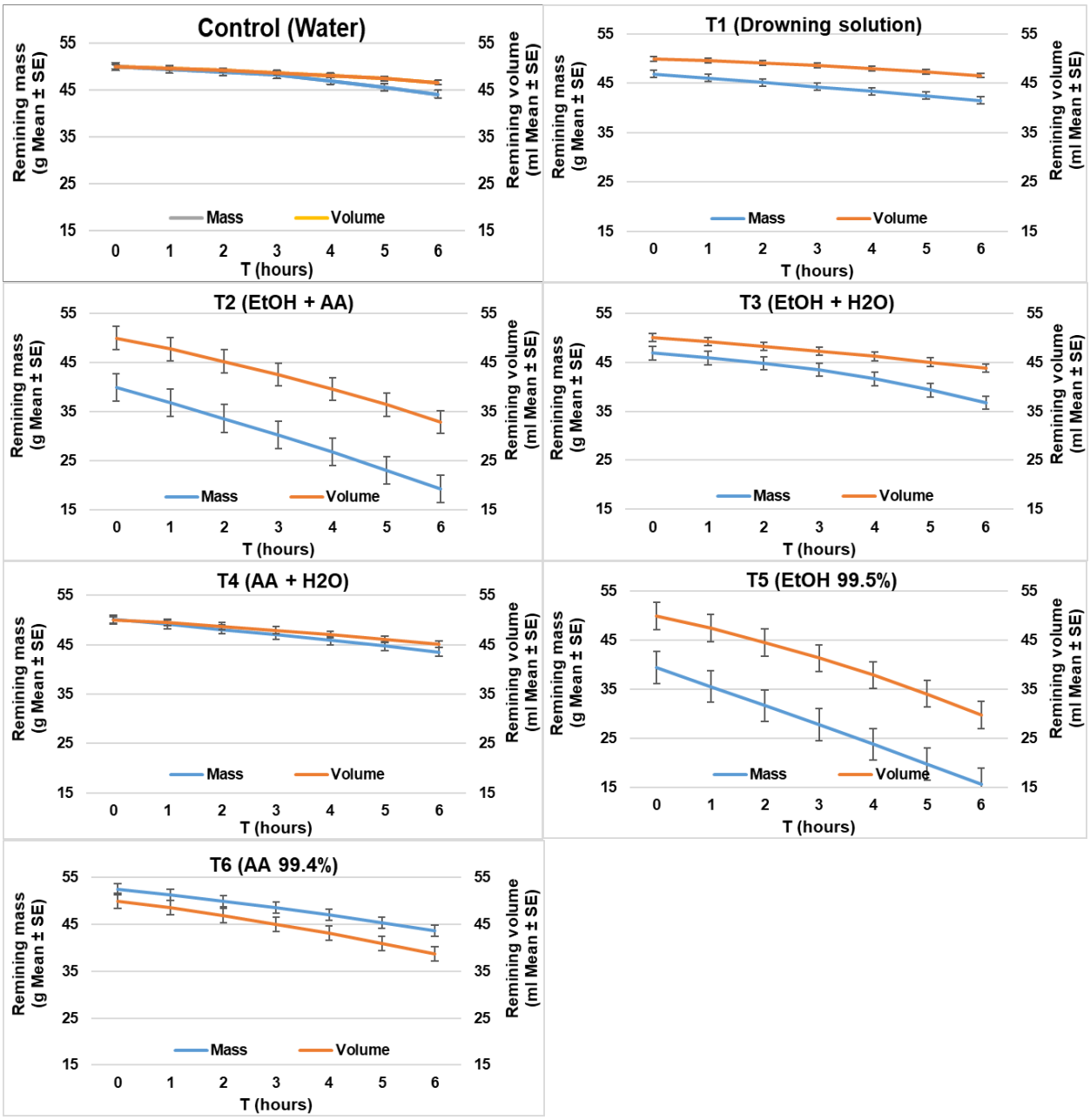


Figure 4. Loss of solution from 50 ml flask in Experiment 2. The left y-axis is the loss of mass (blue line) and the right y-axis is the loss of volume (red line).

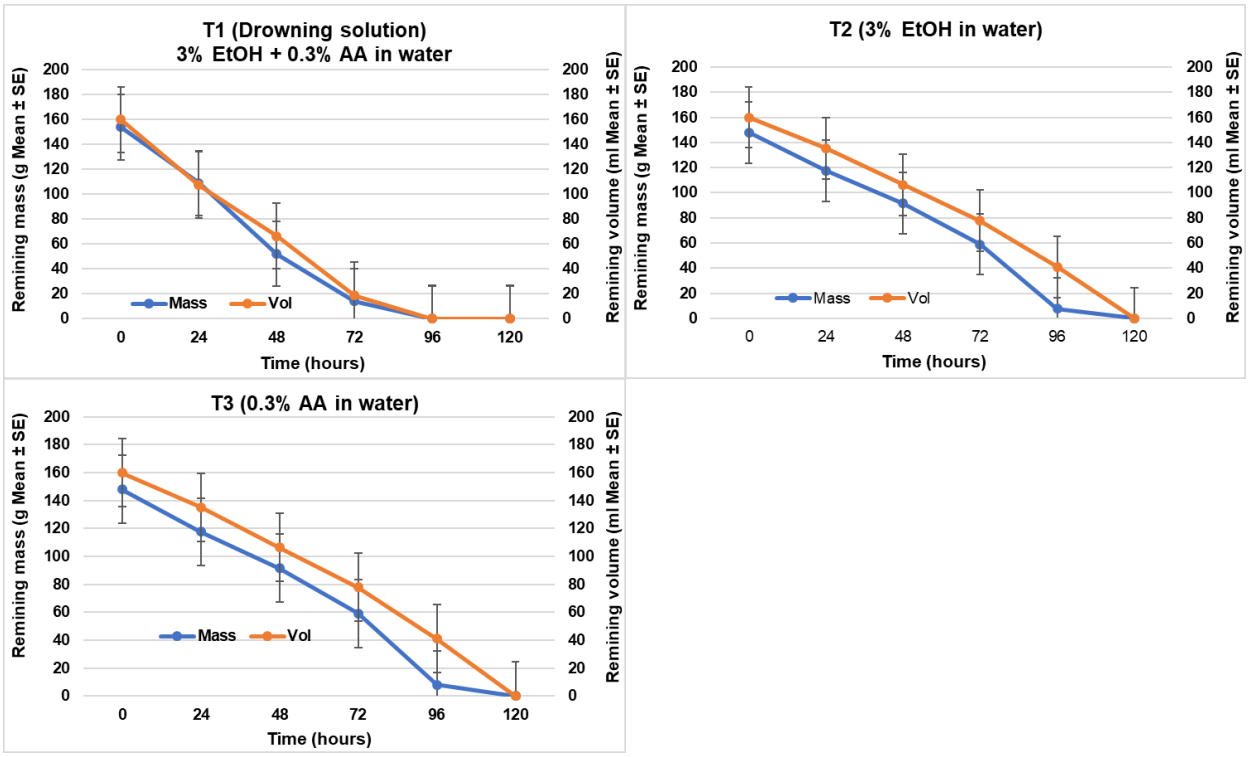


Figure 5. The rate of mass and volume loss in Experiment 3. The left y-axis is the loss of mass (blue line) and the right y-axis is the loss of volume (red line).

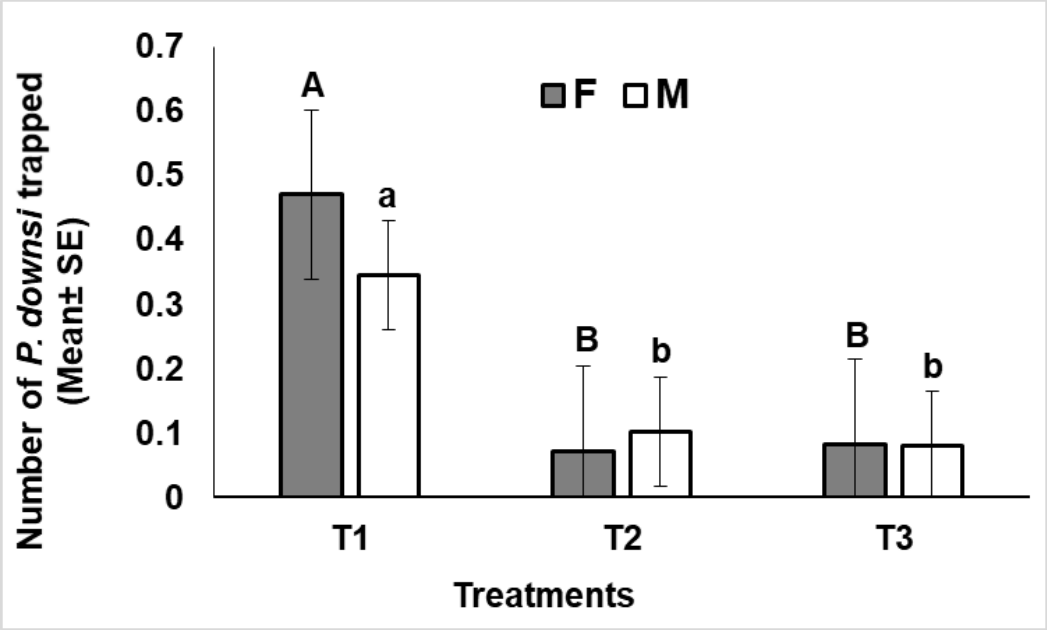


Figure 6. Mean (± SE) numbers of *P. downsi* adult female (F) and male (M) flies captured in experiment 6 . Treatment 1 (drowning solution of 1% AA + 3% EtOH + 96% water) contrasted with treatments 2 (1% AA + 10% EtOH + 89% water in CIPB plus drowning solution of water) and 3 (1% AA + 10% EtOH + 20% propylene glycol + 69% water) indicating significant differences. Statistical tests were based on square-root transformed data. Means from untransformed data are shown. For each treatment, different letters on bars within sex indicate significant differences Tukey-Kramer tests at $P < 0.05$.

CHAPTER IV

Identification of potential semiochemicals for the monitoring and control of *Philornis downsi*

To be submitted to J Chem Ecol.

INTRODUCTION

The parasitic fly *Philornis downsi* (Dodge & Aitken 1968), in its larval stage, feeds on nestling passerine blood causing high rates of mortality in several species of Galapagos land birds (Fessl et el., 2018).

One of the main effects is anemia which has been associated with the mortality of the critically endangered Mangrove Finch chicks (*Camarhynchus heliobates*; Fessl et al., 2010) and Small Ground Finch chicks, *Geospiza fuliginosa*, (Dudaniec et al., 2006), among others (Fessl et al., 2018).

In addition, potential alterations in natural selection and changes in reproductive patterns are likely a result of deformities caused by larvae feeding in the nasal cavities (Kleindorfer & Sulloway, 2016; Fessl et al., 2018).

The identification of semiochemicals has led to improved control strategies for some species of hematophagous insects**,** such as *Ochlerotatus taeniorhynchus*, *Glossina spp*, *Anopheles Atropos*, among others (Kline, 2007)**.** Yet, the use of semiochemical-baited traps for monitoring and control of parasitic flies is relatively new.

One example is the mass trapping efforts, which have proved successful in the control of the tsetse fly, *Glossina morsitans*, the vector of African sleeping sickness. Commercial traps have proved useful in the control this parasitic fly (Kline, 2006).

Collignon (2011) initiated the first efforts to identify semiochemicals for *P. downsi*. He documented marked sexual dimorphism in the composition of the cuticular lipids of *P. downsi* following sexual maturation. Immediately following eclosion, male and female cuticular lipids were indistinguishable, but by day five, the composition of male cuticular lipids changed to the extent that they retained only a single compound in common with females.

This suggests that there may be a cost to an immature male in producing the odor of a mature male and therefore a significant benefit in producing the odor signal upon reaching sexual maturity (e.g. mate attraction at the appropriate life stage).

An attractant in the male cuticular lipids could be produced directly, or as a precursor that is oxidized to yield an attractive pheromone as has been reported for some species of Hymenoptera (Stanley-Samuelson & Nelson, 1993). Photo-oxidation of cuticle lipids in *P. downsi* produced 64 compounds in the males and 36 in females (Collignon, 2011). In *M. domestica*, photo-oxidation yielded nine compounds in males and 19 in females.

Cha et al. (2016) found that ethanol and acetic acid produced by yeast fermentation are attractive to *P. downsi* and can be a productive starting point for the development of lures for *P. downsi*. Eliminating the non-attractive and redundant fermentation volatiles greatly increased the species-specificity of the lure; however, some non-target insects were also attracted to traps baited with ethanol and acetic acid.

Also, while the two-component lure is attractive, the compounds evaporate quickly (Cha et al., 2016), so a more species-specific and controlled-release system is desirable for control efforts.

Although the use of attractants with food odors has been widely used especially for the control of fruit fly parasites, the use of pheromones in the integrated control of pests is more advantageous (Cha et al., 2012; Landolt et al., 2012), since pheromones have a greater range of attraction, an advantage of hand-applied dispensers, use in a wide variety of traps, specificity attracting one of the sexes (advantage in mating disruption) and relative low cost (Witzgall et al., 2010).

Many insects release pheromones from glands associated with the reproductive tract. For example, male *Phyllophaga lanceolata* (Coleptera: Scarabaeidae) are attracted to odors released by females while extending their genitalia. Similarly, female *Sanninoidea exitiosa* (Lepidoptera: Sessidae) raise the abdomen and extend their genitalia, releasing a pheromone that attracts males (Jacobson, 1972).

However a previous study conducted by (Collignon, 2011) did not distinguish between compounds present in genitals and cuticular extracts that are possibly masked.

In the order Diptera, our understanding of pheromone use is far from complete. In the early stages of sexual attraction, they may rely primarily on visual cues, such as in tsetse and house flies. Male *Lucilia sericata* (Meigen, 1826) (Diptera: Calliphoridae) respond to the characteristic frequency of reflected light flashes from passing females (178 Hz) and fly from their perch to intercept the female (Eichorn et al., 2017).

Complex communication in some fruit fly species includes nitrogen-containing compounds, terpenes, and aliphatic compounds that compose sexual and aggregation pheromones, and host plant volatiles (Howse et al., 1998).

In muscoid calyptrate flies, the identification of pheromones as stimulating agents in mating and courtship behavior has been studied in Sarcophagidae: *Metopia*

campestris (Wcislo, 1986), Tachinidae: *Gymnosoma rotundatum* (Higaki & Adachi 2011), as well as several species of Calliphoridae (Furukawa et al., 2002).

The present study aims to identify sex pheromones involved in the mate attraction of *P. downsi* from three potential sources: 1) genitalia, 2) cuticular lipids, and 3) photo-oxidized lipids.

METHODS

Insects. Live adult *P. downsi* were collected in McPhail traps installed at El Barranco (0°44'25"S, 90°18'25"W) in the arid lowlands of Santa Cruz Island, Galapagos, Ecuador.

Additionally, larvae and pupae were removed from abandoned and failed nests of the Small Tree Finch (*Camarhynchus parvulus*) and Warbler Finch (*Certhidae olivacea*) in the *Scalesia pedunculata* forest remnants near Los Gemelos (0° 37'34"S, 90°23'10"W) in the highlands (400-700 m.a.s.l.) of Santa Cruz Island.

In order to extract cuticular lipids, genital extracts and perform assays in the laboratories of the College of Environmental Sciences and Forestry of the State University of New York (SUNY-ESF) live pupae and larvae were transported under appropriate permits to the SUNY-ESF in Syracuse, NY, U.S for use in assays.

Adult flies were enclosed in plastic containers (0.47 L), grouped by sex (4-5 flies per container), and maintained in a growth chamber (Percival Scientific, Inc., Perry, IA, U.S.) at 23.0°C, 70% RH, 16:8 L:D, and fed with papaya and blackberry juice, brown sugar, water and milk powder. Adult flies 0-10 days post-eclosion were used for genitalic extracts and electroantennographic (GC-EAD) analysis.

Sample Preparation.

Genitalia extracts (GE) were prepared from field-trapped male and female *P. downsi* adults and from adults that had eclosed in the lab (at SUNY-ESF) from larvae and pupae collected from nests in the Galapagos Islands. The adults that eclosed in the laboratory were sampled seven or more days post-eclosion and were virgins.

Genitalia extracts (males and females separately) were obtained by excising the genitalia and immersing them in 50 µl of hexane in a micro-vial insert for 48 hours.

Cuticular lipids (CL) of both sexes were extracted from virgin adult *P. downsi* that eclosed in captivity (>7 days old) and from adults captured in the field in the Galapagos Islands. The CLs were extracted by immersing live flies in 0.25 ml of hexane for 30-45 seconds.

Oxidation of cuticular lipids.

To identify the active component of the cuticular layer in males of *P. downsi*, CL extracts were placed in a screw-top quartz cuvette (5 cm x 1 cm x 1 cm) and evaporated to dryness under a stream of nitrogen. The sample cuvettes were sealed with a Teflon-lined septum.

The sample vials were placed at 50 cm distance in front of the light beam of a solar simulator (Model 10500, ABET Technologies, Milford, CT, U.S.), which radiates full-spectrum light at wavelengths between 300 and 1100 nm.

To photo-oxidize the compounds in the CL extracts, the samples and their respective controls with methylene chloride, were exposed to light produced by the solar simulator for 24 hours.

Volatile products of photo-oxidation were trapped on activated charcoal sorbent (ORBO32-small, Supelco Inc., Bellefonte, PA, U.S.) by forcing charcoal-filtered air (8 mL/min) into the cuvette through the tip of a disposable pipette piercing the cuvette septum and out of the cuvette, through a second pipette containing the charcoal

sorbent. After collecting the volatiles for 24 hours, trapped compounds were eluted from the adsorbent with methylene chloride (100 µL).

Chemical Analysis.

The genitalia extracts, cuticular lipids, and photo-oxidized volatiles were analyzed using coupled Gas Chromatography-Mass Spectrometry (GC-MS). Analyses were carried out with an Agilent 5975C MSD coupled with an Agilent 7890GC equipped with a DB-WAX capillary column (30 m × 0.25 mm ID, 0.25 µm film thickness; Agilent Technologies) or an HP-5 capillary column (30 m × 0.25 mm ID, 0.25 µm film thickness; Agilent Technologies).

The carrier gas was helium (1.0 ml/min constant flow). The GC temperature program was the same as used for the GC-EAD analyses described below. The MS transfer line was held at 250 °C for DB-WAX or 280 °C for HP-5 ms columns; the MS source was 230 °C; the MS quad was 150 °C.

Mass spectra were taken in EI mode (at 70 eV) in the range from 40 m/z to 350 m/z with a scanning rate of 2.36 scan/s. GC-MS data were processed with the MDS-Chemstation software (Agilent Technologies).

Electroantennography (GC-EAD).

To perform electroantennography analysis, a gas chromatograph (Hewlett-Packard 5890 series II), equipped with a DB-Wax capillary column (30 m X 0.25 mm ID, 0.25 µm film thickness, Agilent Technologies, Wilmington, DE, U.S.) was used.

The injector was set to splitless mode at 250°C with 1 minute hold time. The oven temperature was programmed for five minutes at 40°C; then the temperature was increased 15°C/min until it reached 250°C and then held for five minutes. The carrier gas was helium with a constant flow rate of 2 ml/min.

The column effluent was split 1:1 by a glass Y-connector (Supelco, Bellafonte, PA, U.S.) with nitrogen added as make-up gas (8 ml/min) using another glass Y-connector. Half of the effluent passed to the flame ionization detector (FID) (280°C) and the other half passed through a heated (280°C) transfer line through the wall of the GC oven to a modified condenser tube (60 mm ID X 50 cm long).

A stream of cooled, humidified air (1 L/min) passed through the condenser, combined with the column effluent flowed over the antennal preparation.

The head and prothorax of an adult *P. downsi* were mounted at one end of an acrylic holder (Figure 1) filled with saline solution (7.5 g NaCl, 0.21 g $CaCl_2$, 0.35 g KCl, 0.20 g of $NaHCO_3$, 1L of H_2O). A glass capillary tube (1 mm dia) filled with saline was placed in contact with the third antennal segment.

Gold wire electrodes were inserted into the holder close to the back of the fly's head (indifferent electrode) and in the glass capillary (recording electrode). The output signal from the electrodes was amplified with the Syntech IDAC 79199 serial-data acquisition controller and software (v1.2.4) with a low cut-off (0.05 Hz) and a voltage range of 0-312.5 mV.

GC-EAD analyses were performed on wild caught male and female cuticular lipid extracts, male and female genitalia extracts, and based on results obtained in Collignon (2011), showing that compounds of cuticular hydrocarbons change remarkably during

the maturation of males unlike females of *P. downsi*, we also included results of photo-oxidized male cuticular lipids and the response of females to these compounds.

Y-tube olfactometer assays.

Olfactory preferences of adult wild caught female and male *P. downsi* to test stimuli of genitalia extract of wild caught male and females were assayed in a vertically oriented Y-tube glass olfactometer (23 mm i.d.; stem length 25 cm; arm length 20 cm; arm angle 70°) at 25-28°C and 40-70% RH. Air was pulled through the Y-tube at ~1 L/min with a vacuum pump.

Odor stimuli and controls were placed in separate Erlenmeyer flasks (250 mL) with a two-hole rubber stopper; charcoal-filtered air entered the flask through one hole and passed through vinyl tubing to an arm of the olfactometer. For each replicate, the position of the odor source was randomly assigned to Y-tube arms.

A single broad-spectrum fluorescent light tube was centered 15-20 cm above the olfactometer. Flies were released individually into the stem, flew upward toward the light, and then flew into one or the other olfactometer arms depending on their odor preference. A fly that penetrated ≥ 10 cm into one arm within one minute was recorded as a responder.

All non-responding insects were excluded from statistical analyses. Each replicate employed a new odor source and fly. The Y-tube was rinsed with isopropyl alcohol and dried between replicates.

Field trials.

To determine which sex produces pheromone, we devised a trapping system that utilized either live, caged adults or solvent extracts of adults that could release pheromone and attract wild flies into the traps. We inserted the base of a quartz tube (8 cm long x 3.5 cm dia; closed top, open bottom) (Figure 2a) into the lure receptacle in the top of ball traps (ISCA Technologies, Inc., Riverside, CA, U.S.).

Quartz was used because it allows the passage of more than 90% of UV light (Golimowski and Golimowska 1996; Bender et al. 2000) which is required in some insects to photo-oxidize cuticular lipids. In the live fly experiment, flies were placed inside the quartz tube with sugar water.

For the live fly experiment, the top of the quartz tube was covered with white paper to minimize heat and dehydration stress caused by exposure to full sun (Figure 2b) while still allowing sunning behavior in the lower portion of the tube. In the experiments testing solvent extracts, the vials with the extracts were placed inside the quartz tube with the lid partially open to promote release of the volatiles.

In addition, a new vial was added every day during the three days of the experiment, in order to increase the concentration of the volatiles.

Experiment 1: Field response to live adults.

Dougherty (unpublished data), found that the odor contribution of papaya juice was minimal, but that its behavioral effect on calling adults was potentially significant. Based on this information, we baited McPhail traps with (a) water (negative control), (b)

papaya juice (positive control), (c) one adult male *P. downsi* + papaya juice, or (d) one adult female *P. downsi (age)* + papaya juice.

Fresh papaya juice (1-3 days old; 150 mL) was placed in the bottom of each trap as a food resource because many species of insects commonly 'call' from adult or larval food resources. Previous reports (Lincango & Causton, 2009) suggest that papaya juice becomes gradually more attractive to adult *P. downsi* again after 4 to 5 days.

Experiment 2: Field response to genitalia and cuticular lipid extracts.

Field assays were conducted to determine the source of the semiochemicals in Experiment 1. We tested solvent extracts of cuticular lipids and genitalia.

We baited McPhail traps with (a) cuticular lipids from field-caught female adults (**CLFF**), (b) cuticular lipids from virgin female adults emerged in the lab from field-collected pupae (**CLLF**), (c) cuticular lipids from field-caught male adults (**CLFM**), (d) cuticular lipids from virgin male adults emerged in the lab from field-collected pupae (**CLLM**), (e) papaya juice (positive control), (f) genitalia of field-caught female adults (**GFF**), (g) genitalia of virgin female adults emerged in the lab from field-collected pupae (**GLF**), (h) genitalia of field-caught male adults (**GFM**), or (i) genitalia of virgin male adults emerged in the lab from field-collected pupae (**GLM**), all treatments were tested with a combination of fresh papaya juice.

Experiments one and two were carried out in El Barranco (0°44′34.1″S, 90°18′10.4″W), an arid lowland site and Los Gemelos (0° 37'34"S, 90°23'10"W), a humid highlands site, on Santa Cruz Island.

The traps were installed at a height of between four and five meters near the top of the forest canopy, and the trials with modified McPhail traps with quartz tubes were carried out between April 22 and May 23 of 2014 and February 3 and May 1 of 2014, while the tests with extracts of cuticular lipids and genitals were carried out between April 26 and May 15 of 2017.

Traps within and among replicates were separated by at least 10 m. Treatments were arranged in a randomized complete block design. Traps were checked every 24 hours; all flies in the traps were collected, identified, and separated by sex.

Each test replicate was conducted for three consecutive days; then trap placement was rotated to account for potential position effects. The papaya juice, live flies and extracts were replaced and then a new three-day replicate commenced.

Statistical analysis.

For all studies, a randomized complete block design with 10~45 replicates were used. The fly capture data were transformed into square root to improve normality and homoscedasticity. However, the GLM function for a Poisson distribution with log link was used when transformation did not improve normality and homoscedasticity.

For all experiments, the treatment means were compared using the Tukey-Kramer test ($\alpha = 0.05$). The analyses were carried out using the statistical package R version 3.1.3 (R Core Team 2013) and R-Commander package (Fox & Bouchet-Valat, 2018).

RESULTS

Oxidation of cuticular lipids.

Photo-oxidation of male cuticular lipid extracts from field-caught adult males resulted in the formation of several new components all of which eluted earlier than the parent lipids (Figure 3). The compounds that changed their relative quantities or formed after photo-oxidation are listed in Figure 4.

GC-EAD analysis detected 18 compounds in the photo-oxidized male samples that consistently elicited antennal response from virgin female antennae (n=4), compounds that elicited the strongest responses were: 3-hexanone, 2-hexanone and 1-methylcyclopentanol (Figure 5).

Chemical Analysis.

Distinct differences were observed between field-caught male and female genitalia extracts (Figure 6). Male extracts are characterized by methyl-branched and unsaturated hydrocarbons (Figure 6a), while female genitalia extracts contained substantial quantities of fatty acids (Figure 6b) as well as compounds eluted from nonacosane to hexatriacontane.

Y-tube olfactometer assays.

A total of 178 olfactory choice assays were conducted including all four reciprocal tests in response to olfactory preferences of wild caught *P. downsi* female and male adults to stimuli of genital extract of males and females.

Significant deviation from random outcomes (i.e., 50/50) occurred only for wild-caught females responding to wild-caught male genitalic extracts. The binomial test showed that the response of wild-caught females to male genitalic extracts was significantly different from random chance (P = 0.02) Figure 7.

Field trials

Field response to live adults.

A total of 136 flies (53 males and 83 females) were captured (Figure 8). The treatment containing a live male in the presence of papaya juice attracted more *P. downsi* females than papaya juice alone, papaya juice + a female, or water alone.

The generalized linear model detected significant differences among treatments (Poisson regression, X^2=183.63, df=4, P< 0.001), and differences between females and males captured (X^2=6.672, df=1, P< 0.05). Treatment C (live male plus papaya) captured significantly more female and male flies (Tukey–Kramer tests at P < 0.05).

Test showed no significant differences in the number of *P. downsi* males captured in the four treatments. However, the number of females captured in treatment **C** was high in contrast to three other treatments (Tukey-Kramer test at P < 0.05).

Field response to genitalia and cuticular lipid extracts.

A total of 1,671 flies were captured at El Barranco (1,076 females and 595 males). The analyses of the generalized linear model with a Poisson distribution show statistically significant differences between the effects of the treatments (X^2=76.342,

df=1, P< 0.001), and in the capture of females and males (X^2=76.342, df=1, P <0.001) (Figure 9).

Tukey-Kramer test showed no significant differences in the number of *P. downsi* males captured in the eight treatments and one control. However, the number of females captured in the treatment **GFF** (extracts from genitals of field-caught female flies) captured was significantly higher in contrast to the **control** (papaya juice) and treatments **CLFM (**cuticular extracts from field-caught males) and **GLM** (genital extracts from lab-emerged virgin males) (Tukey-Kramer test p <0.05).

DISCUSSION

Although pheromone production by abiotic oxidation of lipids has been documented in several insect orders, this area of study is relatively little explored in insect chemical ecology (Staples et al., 2010).

In Diptera, the production of volatile pheromone components from unsaturated lipids by oxidation has been studied in *Musca domestica* (Dilwith et al., 1981), and more recently by Collignon (2011) who document that the photo-oxidation of male *P. downsi* cuticular lipids produces new compounds suggesting the production of pheromones.

The role of cuticular lipids in chemical communication has been extensively studied in *Drosophila* (Blomquist & Vogt, 2003) but in other Diptera the roles of these compounds have been addressed only sporadically (Stanley-samuelson & Nelson 1993). Sexual dimorphism in cuticular lipid profiles of solitary insects including dipterans often develops along with post-eclosion maturity (Jackson & Bartelt, 1986; Schaner et al., 1989).

Among the multiple sensory and behavioral interactions involved in dipteran mating, female cuticular lipids function as a specific chemosensory conditioned stimulus (Tompkins et al., 1983). In *D. melanogaster*, female cuticular lipid pheromones, which are the specific chemosensory cues that function as the conditioned stimulus in courtship conditioning, stimulate and suppress mating with virgin and mated females, respectively.

This system of chemical signals mediates male preferential mating with virgin females and confirmed the role of CHPs as a component of the conditioned stimulus toward targets releasing certain pheromones (Siwicki et al., 2005).

Abiotic oxidation of cuticular lipids as a mechanism of pheromone production is typically associated with female insects and has been documented in *Acantholyda erythrocephala* (Hymenoptera: Pamphiliidae) (Staples et al., 2010), *Cephus cinctus* (Bartelt et al., 2002), *Pikonema alaskensis* (Tenthredinidae) (Bartelt & Jones, 1983), *Macrocentrus grandii* (Braconidae) (Swedenborg and Jones, 1992) and *Anoplophora glabripennis* (Wickham et al., 2012) and *Musca domestica* (Dilwith et al., 1981).

This is in contrast to both the current study and that of Collingnon & Teale (2011) that suggest that males are the pheromone-producing sex in *P. downsi*. In GC-EAD analyses conducted in this study, 18 compounds from the cuticles of male flies that had been photo-oxidized elicited EAD responses in virgin female flies.

In addition, the largest number of responses was recorded in low–molecular weight volatile compounds, suggesting that the response of females in GC-EAD analysis is due to the formation of volatile oxidized compounds, indicating that oxidation may play a role in converting lipid precursors to active pheromones.

Photochemical oxidation involving UV light, oxygen and cuticular lipids can give rise to compounds not present in the original cuticular lipids (Frankel, 2005). The results of this study and those of Collignon & Teale (2011) show that photo-oxidation of male *P. downsi* cuticular lipids produces new compounds that are potential attractants.

Furthermore, photo-oxidation also alters the concentrations of the parent lipids (Figure 4). Thus, photo-oxidation may result in the same volatile products, but in different proportions (Bartelt et al., 2002).

The live fly field experiment and the y-tube olfactometer assays both support the conclusion that in *P. downsi*, males are the pheromone-producing sex. Although the live

fly field experiment allowed for the possibility of pheromone production by means of photo-oxidation by caging the flies in quartz tubes, it did not exclude the possibility of pheromone release from genital glands rather than directly from the cuticular lipid layer.

Significant attraction of adult females to wild-caught adult males genitalia extracts in the olfactometer experiment indicates that the pheromone is released from the genitalia, though it is still possible that the small amount of cuticle from the terminal abdominal segment included in the extracts was the source of the attractant.

Although the quartz tube field experiment indicated that adult males were attractive to females, the degree of attractiveness is very likely dependent on the age of the males, which influences cuticular lipid composition. Similarly, in females of *M. domestica*, the production and release of certain compounds, mainly monoenes, ketones, epoxides, and dienes, increases with age (Murvosh et al., 1965; Mpuru et al., 2001).

The failure of the field experiment (testing cuticular and genitalia extracts) to detect a significant treatment effect may have been due to the limited quantity of active components in the extracts. Solvent extracts only contain the material present in the insect at the time of extraction; that material (pooled samples of 10 flies) was then placed in the field for three days.

Thus, the failure of most of the extracts to attract more adult *P. downsi* than the papaya juice controls could have been due to the use of too little attractant. Ongoing and future work will make use of olfactometer assays and solvent extracts, which do not have these problems.

The production and release of insect sex pheromones is often associated with genital glands. In many insects, including certain Coleoptera and Diptera, epidermal glands that secrete cuticular lipids are another source of pheromone production (Schal et al., 1998). Several species are capable of selectively transporting shorter-chained hydrocarbon pheromones and pheromone precursors to the pheromone gland in the abdomen.

However, a clear comprehension of how the hydrocarbons are transported up to the cuticle and its disposition in the surface of the insects is still unknown (Blomquist & Bagneres, 2010). Nevertheless, Schal et al. (1998) suggest that glands associated with cuticular lipids are often linked with other parts of the body involved with pheromone transport.

The role of the genitalia in insect chemical communication suggests a stimulation function. For example, in some species of beetles such as *Phyllophaga lanceolata*, males can feed on the same leaf with a female; however, they are not attracted to the female until she extrudes her genitalia scattering pheromones (Jacobson, 1972).

Additionally, the attraction of sex pheromones produced in the genitalia has been investigated in the common house fly, *Musca domestica*. In petri dishes, males jumped on models treated with virgin female genitalia extracts and attempted to copulate with them (Jacobson, 1972).

The use of behavior-modifying odors may give the conservation biologist a useful tool when investigating methods to protect species threatened by parasitic insects. Yet, despite the growing interest in conservation biology, and in particular endangered

species protection, it is striking that little effort has been made to study chemical communication (Svensson et al., 2004).

In this sense, the practical objectives of our research on *P. downsi* semiochemicals are focused on potentializing and using these compounds in control programs for the monitoring and reduction of populations of this invasive species. In particular, our results demonstrate the usefulness and versatility of pheromone lures for monitoring and trapping *P. downsi*.

CONCLUSION

The detection of invasive species by using pheromones or other chemical attractants is an established practice in applied entomology (Witzgall et al., 2010). In the context of conservation, the potential long-term benefits of monitoring and population suppression make semiochemically-based management cost effective, even when the semiochemicals require custom synthesis (Larsson & Svensson 2009).

The efficiency of pheromone-based management stems from the advantage of selective exploitation of the species-specific communication channel used in mate attraction (Svensson et al., 2012).

The combined use of GC-EAD and laboratory and field behavioral assays has contributed significantly to increasing our understanding of the chemically mediated behaviors in the courtship and mating system of *P. downsi*. The live fly field experiment and the laboratory assays demonstrated that males are the pheromone-producing sex in *P. downsi*.

Furthermore, we have evidence that the pheromone is released from the cuticular lipid layer. Additional work is required to verify the anatomical source of this chemical signal. Currently, we have evidence that cuticular lipid extracts of male genitalia are attractive, although there could be other cuticular sources in intact male flies.

Regardless of the origin of the cuticular lipids, this work shows that photo-oxidation is required to activate the pheromone. Ongoing work is aimed at identifying the active components of the cuticular and genitalia extracts, in order to synthesize and use them as lures to massively capture *P. downsi*.

The development of monitoring systems for individual species such as *P. downsi* represents a comparatively large initial investment, but offers unique knowledge about model species. The efforts used in early detection with food odor attractant and disruption of pheromone parasitism make this conservation-monitoring method with semiochemicals cost effective, even when the compounds need to be custom synthesized.

Monitoring with food odors and pheromones represents an advantage in the selective apprehension of *P. downsi*, exploiting the same communication channel and usually taking advantage of selection by sex. Furthermore, the detection of alien species by using pheromones or synthetic chemical attractants is a rapidly developing field in applied entomology.

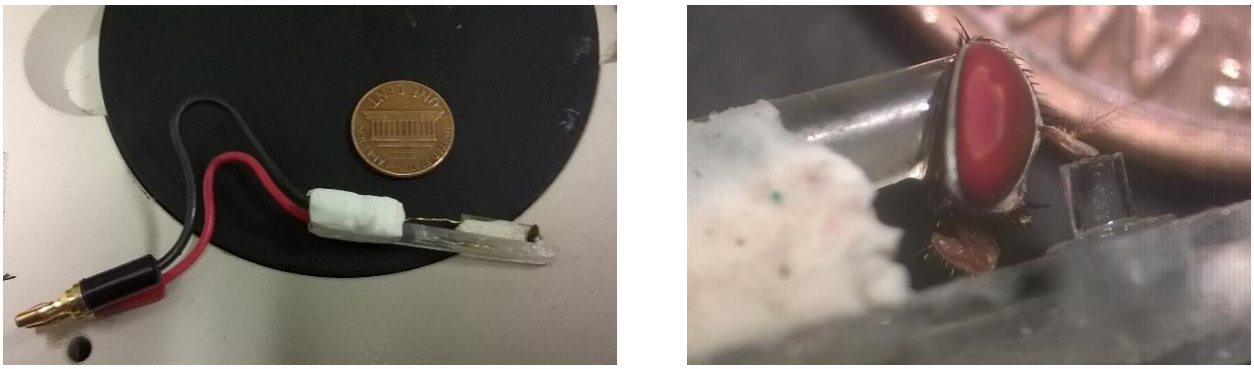


Figure 1. Antennal preparation for coupled gas chromatography-electroantennography (GC-EAD). Entire set up (left), and close-up (right).

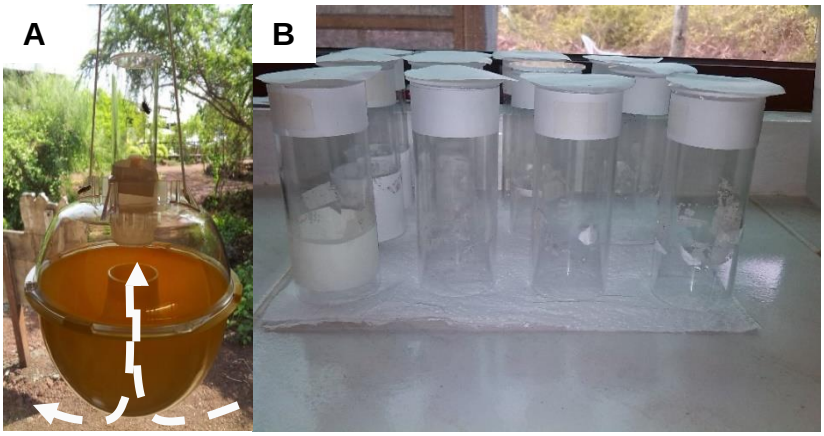


Figure 2. Modification of ball traps used to test attractiveness of male and female *P. downsi* and tissue extracts. (A) Quartz tube installed in the lure receptacle of a ball trap. (B) Paper covers on the quartz tubes to decrease internal temperature.

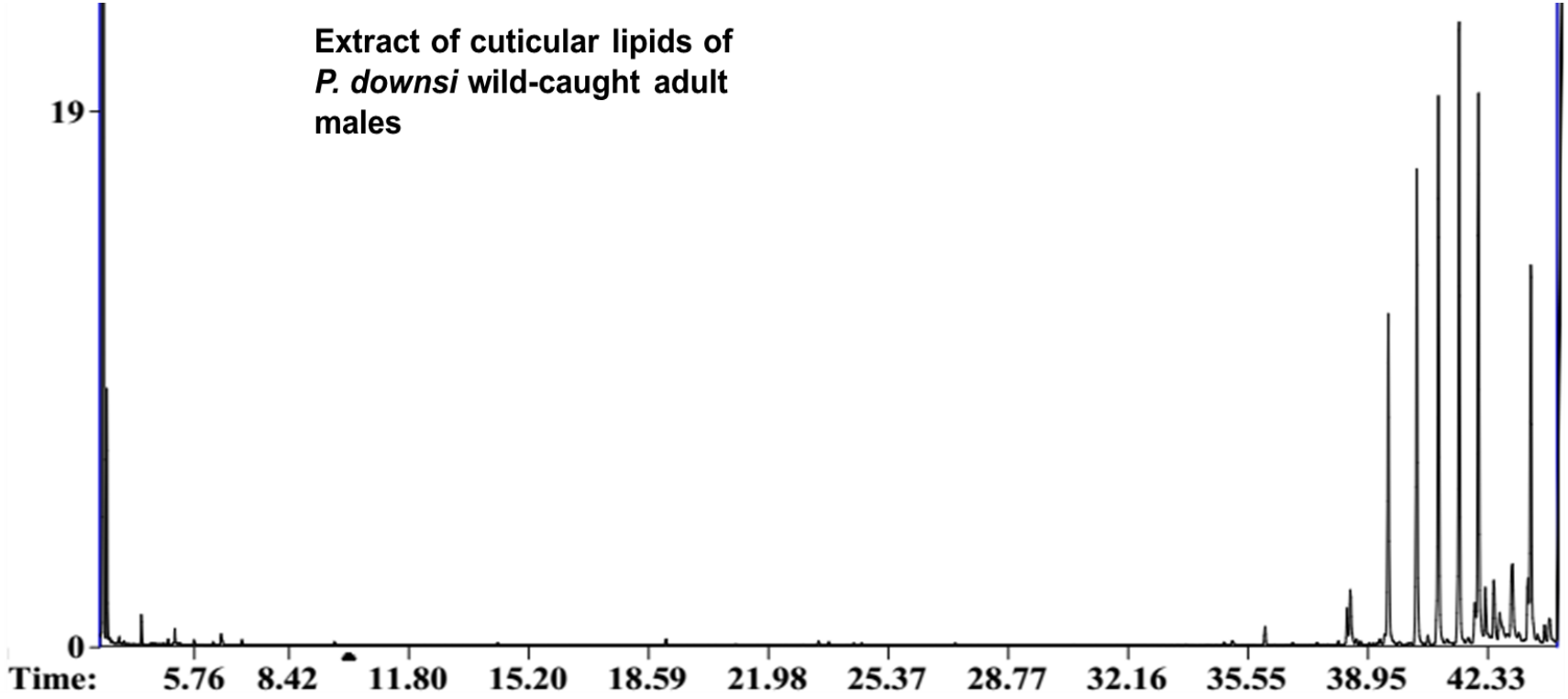
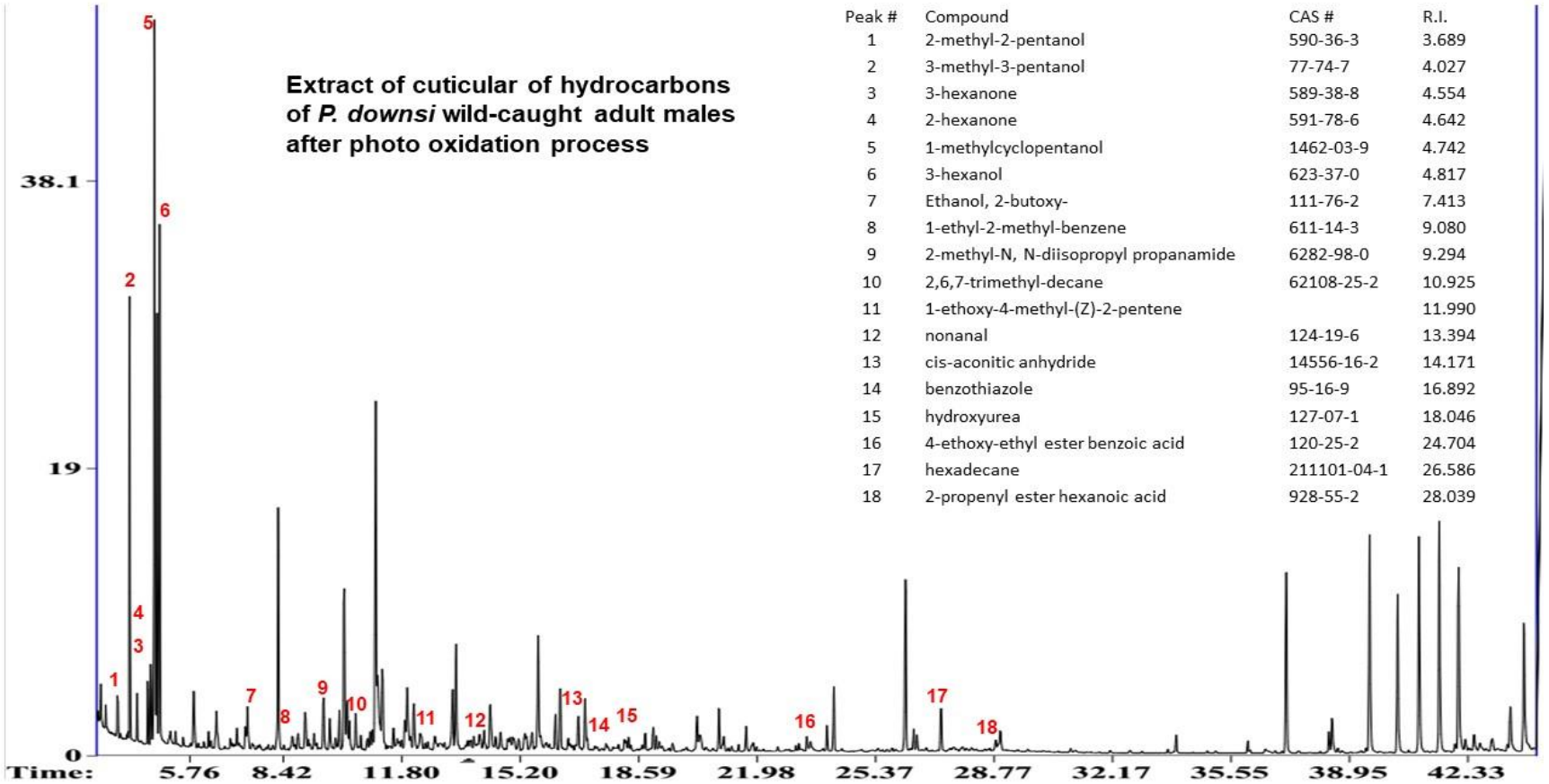


Figure 3. Total ion chromatograms (TIC) of cuticular lipid extracts of *P. downsi* wild-caught adult males.



Peak #	Compound	CAS #	R.I.
1	2-methyl-2-pentanol	590-36-3	3.689
2	3-methyl-3-pentanol	77-74-7	4.027
3	3-hexanone	589-38-8	4.554
4	2-hexanone	591-78-6	4.642
5	1-methylcyclopentanol	1462-03-9	4.742
6	3-hexanol	623-37-0	4.817
7	Ethanol, 2-butoxy-	111-76-2	7.413
8	1-ethyl-2-methyl-benzene	611-14-3	9.080
9	2-methyl-N, N-diisopropyl propanamide	6282-98-0	9.294
10	2,6,7-trimethyl-decane	62108-25-2	10.925
11	1-ethoxy-4-methyl-(Z)-2-pentene		11.990
12	nonanal	124-19-6	13.394
13	cis-aconitic anhydride	14556-16-2	14.171
14	benzothiazole	95-16-9	16.892
15	hydroxyurea	127-07-1	18.046
16	4-ethoxy-ethyl ester benzoic acid	120-25-2	24.704
17	hexadecane	211101-04-1	26.586
18	2-propenyl ester hexanoic acid	928-55-2	28.039

Figure 4. Total ion chromatograms (TIC) of cuticular lipid extracts of *P. downsi* wild-caught adult males after photo-oxidation.

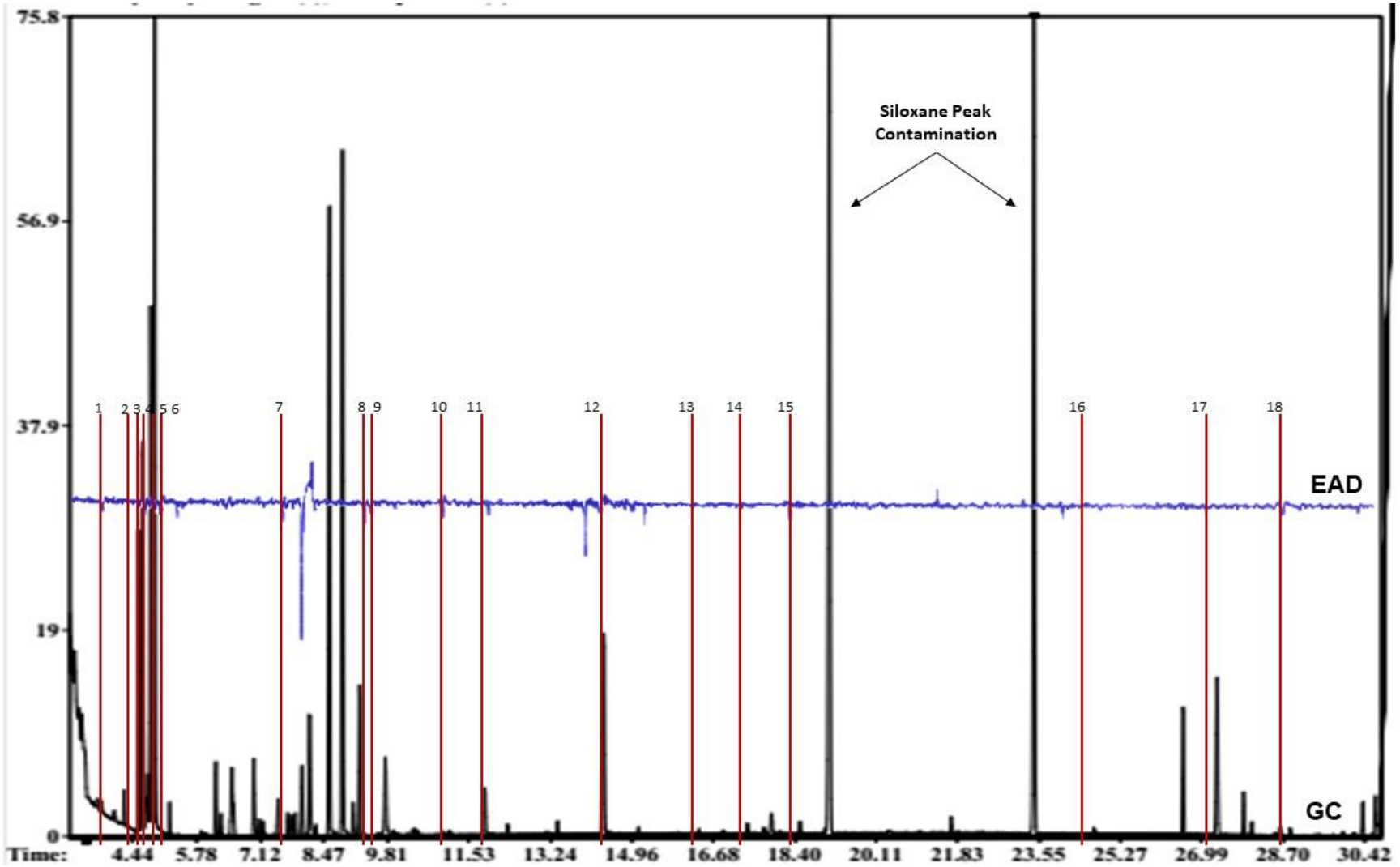


Figure 5. Recording of antennal responses of *P. downsi* virgin females (blue: 'EAD'), elicited by components of photo-oxidized extracts of cuticular lipid extracts from *P. downsi* wild-caught adult males (black: 'GC').

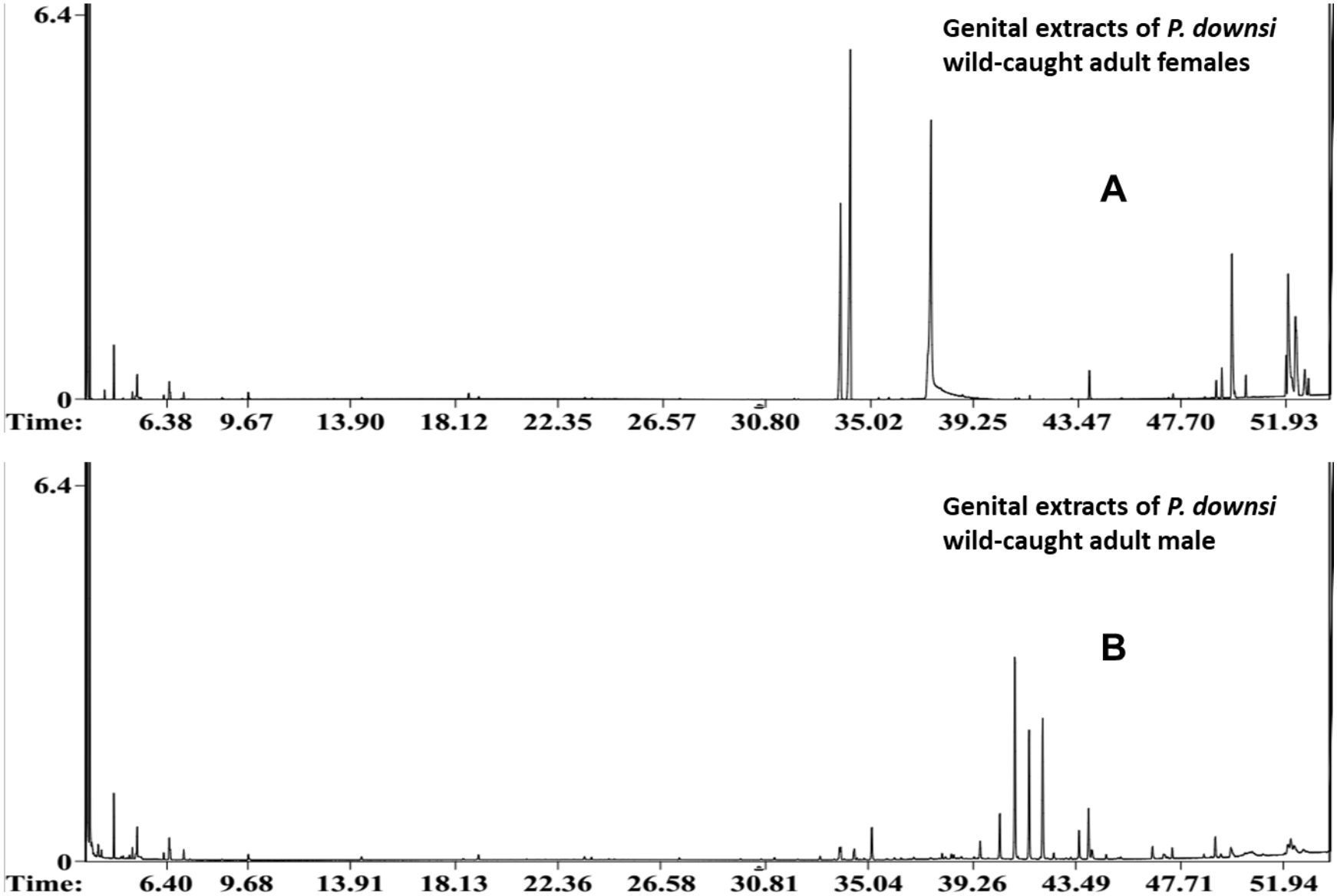


Figure 6. Total ion chromatograms (TIC) of extract of genitalia from wild-caught adult females (A) and males (B).

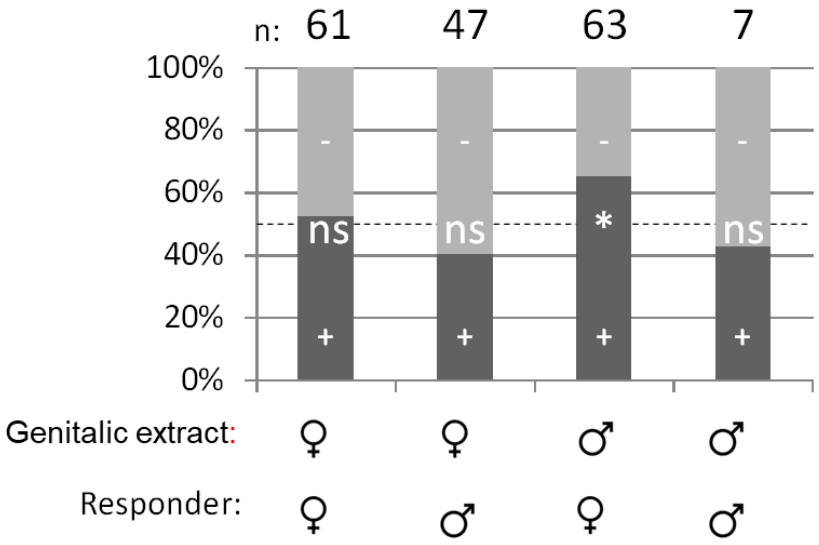


Figure 7. Olfactory preference responses in Y-Tube olfactometer performed with wild caught *P. downsi* adult females and males reared in the laboratory, responding to stimuli from the genitals extract of wild caught males and females.

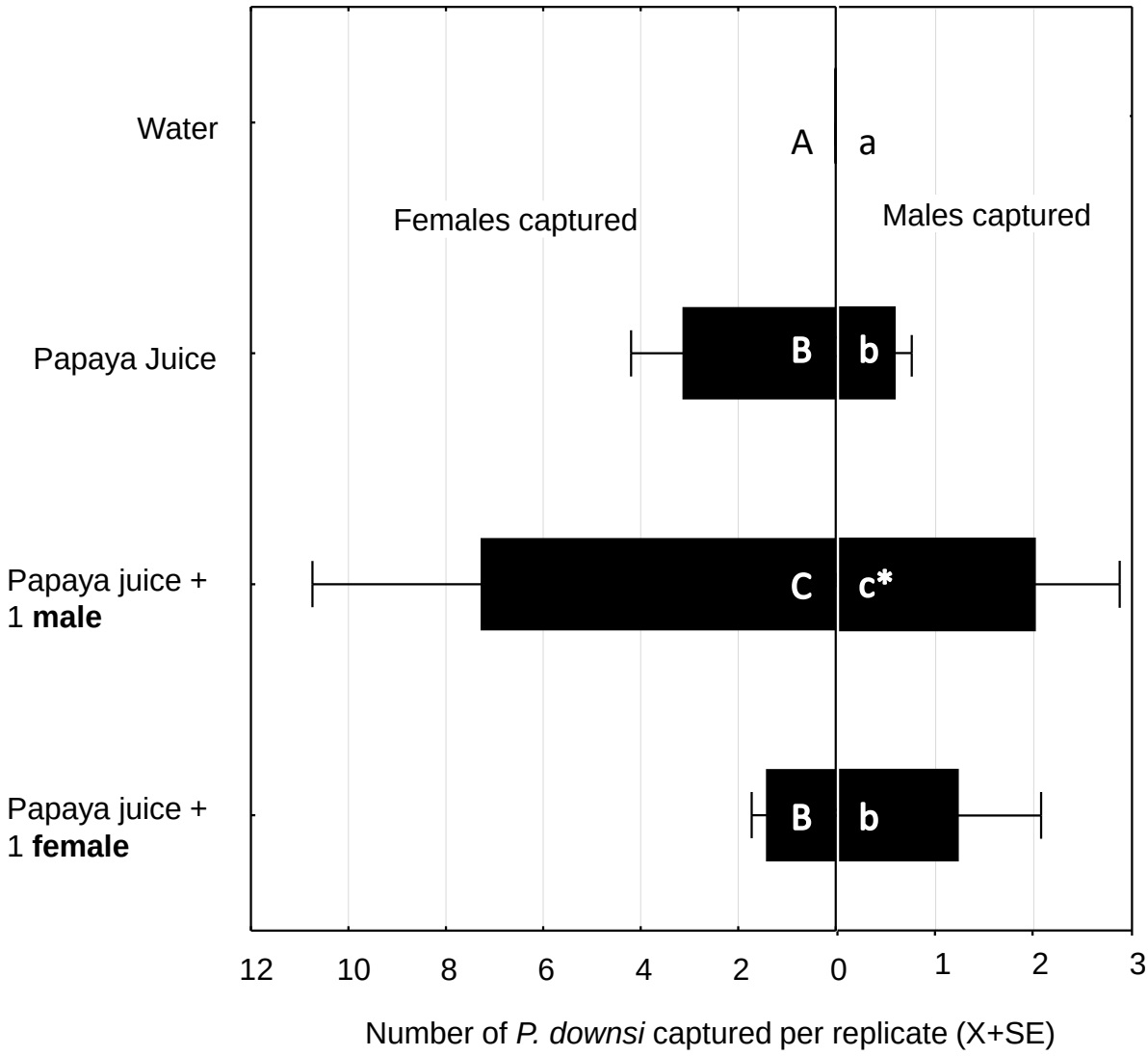


Figure 8. Mean (± SE) number of *P. downsi* caught with live flies with papaya juice. The letters of the treatments indicate significant differences calculated with the Tukey-Kramer test at P <0.05.

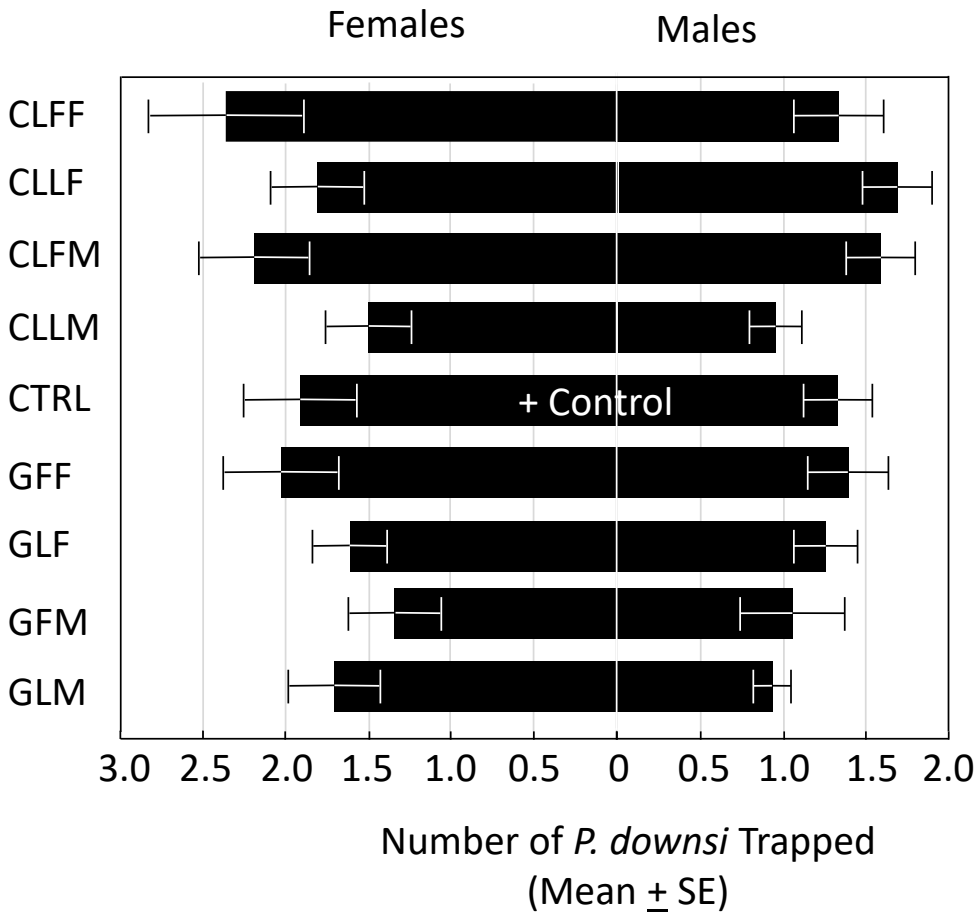


Figure 9. Mean (± SE) number of flies captured with baits of cuticle lipid extracts (CL) and genital extracts (G) of field-captured (F) and virgin adults eclosed in the laboratory (L) female (F) and male (M) flies (E.g. CLFF is the abbreviation for Cuticular Lipid extracts of Field-collected adult Females).

CHAPTER V

CONCLUSIONS

Insects that feed on vertebrate blood are difficult to control. Investigating the ways in which hematophagous insects interact with one another, their environment, and their hosts is providing valuable insights that will lead to the development of improved control technologies.

For example, the recent advances in chemical ecology research have led to the identification and synthesis of new semiochemicals with great potential for use in lures as monitoring and control tools against hematophagous insects (Logan and Birkett 2007).

Like other insects, flies – and most likely *P. downsi* – use sex pheromones as part of their mating behavior (Howse et al., 1998). On the other hand, it has been shown that during periods without reproductive activity, flies often produce aggregation pheromones to bring other individuals of the same species to food sources (e.g. fermenting fruit) or a host (Bartelt et al., 1985; Howse et al., 1998).

The use of semiochemicals by adult *P. downsi* is apparently complex and the exact mating sequence and the semiochemicals that are involved remain largely unknown. Kairomones produced by the host bird during nest building, egg laying, incubation, brooding, and feeding of the chicks, as well as sex or aggregation pheromones are involved in mate location and subsequent parasitism (Fessl et al., 2006; Kleindorfer and Dudaniec 2016; Dudaniec et al., 2010).

Although it has not been possible to directly observe the interactions between female and male *P. downsi* in the field, it is presumed that certain environmental conditions, such as temperature, humidity, wind direction, distance between flies and hosts, and visual cues are likely to be essential factors affecting the reproductive behavior of *P. downsi* (Kuramochi, 1989).

Mass rearing *P. downsi* is crucial to the development of the sterile insect technique and for the purpose of producing the insects for research use. Yet, mass rearing has been challenged by the fact that the conditions of natural reproduction in the field do not resemble those created artificially in the laboratory.

A significant part of the difficulty is with low mating rates in the laboratory. Thus, the results of chemical ecological studies may also provide insight to the mating sequence of *P. downsi*.

The horn fly, *Haematobia irritans* (Muscidae), is a good example of the complexities involved in the reproductive behavior and parasitism of parasitic flies of this family. The reproductive behavior of the horn fly includes a series of semiochemical signals that include kairomones, sex and aggregation pheromones, as well as a complex system of visual signals used by both sexes before and during mating (Kuramochi, 1989.

Comparing the evidence presented in this thesis with the mating system of the horn fly, I hypothesize a sequence of semiochemically mediated adult behavior including feeding, host location, mate location, mating and oviposition (Figure 1).

However, validation of the hypothesized sequence will require future research to verify the steps of the sequence and to elucidate the attractive kairomones and

pheromones involved. An important aspect of the identification of these semiochemicals is the component blends and blend ratios.

My results suggest that food odors and pheromones derived from virgin female genitalia as well as male cuticular hydrocarbons potentially contain chemical signals that are vital to the reproductive behavior of *P. downsi*.

On the other hand, there is the possibility that some signals that stimulate mating and parasitism or oviposition behavior come from the pupal stage of *P. downsi*, as seen in *Musca domestica*, where the stimulus to lay eggs comes from semiochemicals produced by previous clutches of conspecifics (Zvereva, 1984).

The importance of feeding and food odors in mate searching cannot be overstated. Studies carried out on *M. domestica* suggest that feces are a rich source of proteins for adults and are actively sought out (Lam, 2010). Likewise, in *P. downsi*, feeding on fermenting fruit is likely important in obtaining the energy resources needed for mate-searching, host-searching and egg production.

The hypothesized mating sequence and subsequent oviposition of eggs by *P. downsi* begins with feeding then progresses to the location of the host nest by males. Once the females and males have fed, both females and males locate the hosts via kairomones and park near nests before mating.

Pheromones and host kairomones then stimulate mating. Mating occurs after mate location by visual cues (possibly fluttering of wings) and sexual or contact pheromones (Figure 1).

Once the mating sequence of *P. downsi* has been confirmed and the semiochemicals mediating the steps of the sequence have been identified, artificial

rearing will be enhanced by the understanding of the cues required for mating. Additionally, the use of semiochemicals, whether food or host kairomones or sex or aggregation pheromones, will enable efficient trapping with synthetic lures that will surpass that of natural baits.

This will make possible established applications including semiochemically based monitoring, mass trapping and mating disruption.

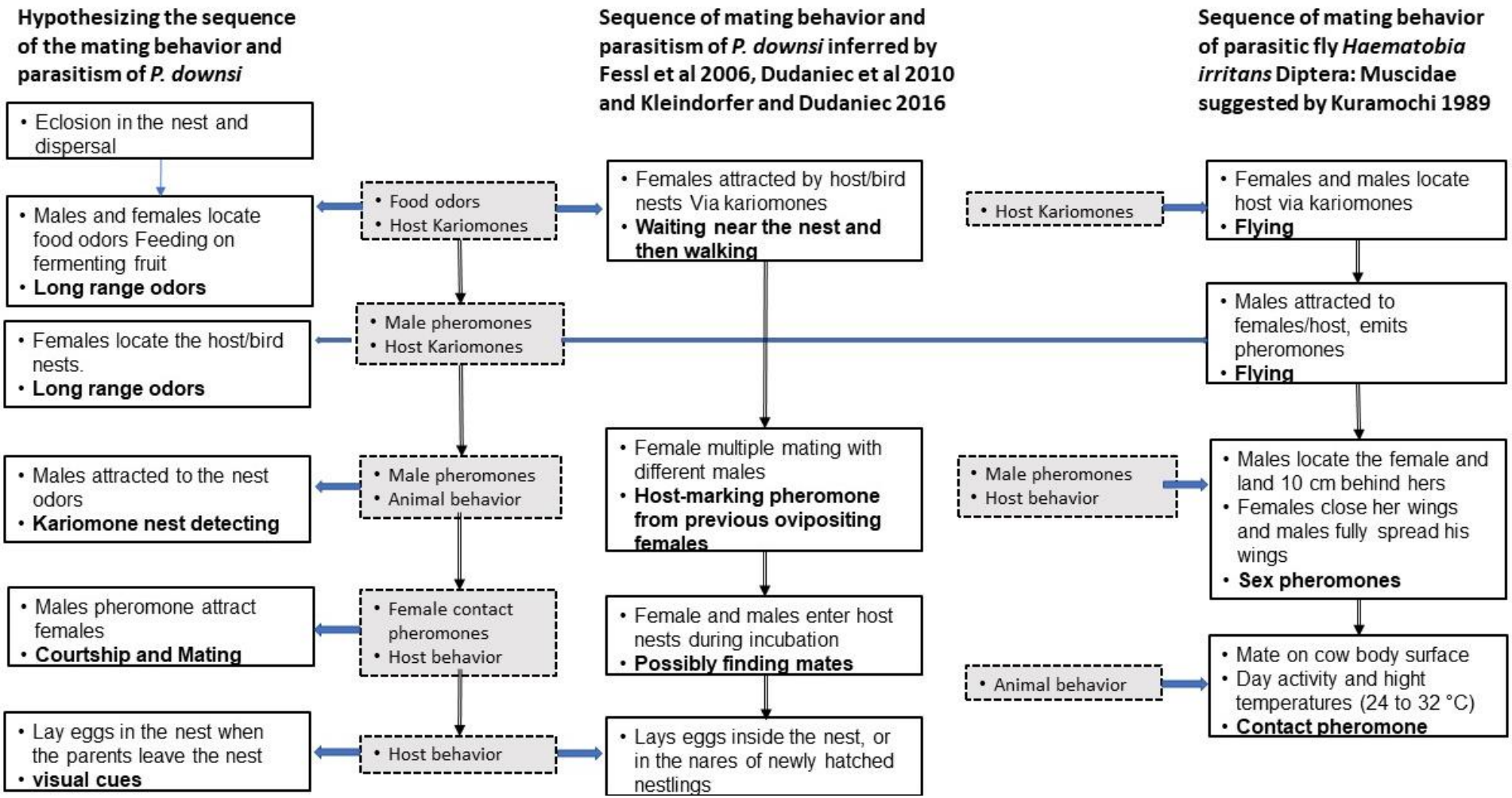


Figure 1. Hypothesized mating sequence and parasitism and the semiochemicals that mediate the five steps in the parasitic fly *Philornis downsi* (left), and comparison with observations derived from Kleindorfer and Dudaniec (2016), Dudaniec *et al*., (2010) and Fessl *et al*. (2006; middle), and the hypothetical four-step mating sequence of *Haematobia irritans* proposed by Kuramochi (1989; right).

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Employment

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