Ethological control of the Mediterranean fruit fly *Ceratitis capitata* (Wied.)

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Cover: Medfly (*Ceratitis capitata*) adult feeding on decaying fruit
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Abstract

The Mediterranean fruit fly *Ceratitis capitata* (Diptera: Tephritidae) is a serious threat for the Bolivian fruit sector. Despite its importance, few efforts have focussed on the development of alternative techniques to broad spectrum pesticides, especially those that suppress the pest using odour-mediated behavior manipulation. In this thesis, I explored whether such odor-based control techniques, using locally available attractants and repellents, could be developed. In laboratory and field trials, ‘chicha’, a corn-based fermented beverage was more attractive than commercial baits to *C. capitata*. However, non-target insects constituted a large portion of the trap catches. A series of traps based on waste material were designed and tested in combination with chicha. One prototype was comparable in captures with the commercial Tephritrap, while at the same time being more selective. In parallel, laboratory screening followed by semi-field and field trials were used to evaluate the effect of essential oils on reducing attraction, oviposition and fruit infestation by *C. capitata*. Laboratory tests showed that the essential oils of three aromatic plants, *Cymbopogon citratus, Tagetes filifolia* and *Schinus molle* reduced attractiveness of oviposition substrates. In semi-field experiments these essential oils suppressed oviposition in peach, whereas in a field trial, *S. molle* oil mixed in dollops of biodegradable wax reduced infestation in peach orchards.

Gas-chromatography coupled to electro-antennographic detection (GC-EAD) and electro-palpographic detection (GC-EPD), were used to characterize the volatile profile of essential oils (repellents) and chicha (food attractant) with the corresponding sensory responses to these. The three oils were all dominated by terpenoids and differed substantially in the classes of constituted compounds, their relative amounts and the pattern of antennal/palpal responses. For chicha volatiles, antennal and palpal sensory responses of three tephritid species *C. capitata*, *Zeugodacus cucurbitae*, *Bactrocera dorsalis* and the unrelated *Drosophila melanogaster*, overlapped considerably with volatiles that are conserved across fresh fruits. This lead to conjecture that these may have served as pre-adaptive bridges mediating shifts from ancestral rotting substrates to ripening fruit. However, responses to fermentation volatiles in tephritids also diverged with the ecological niche: specialist *Z. cucurbitae* was most divergent, whereas generalist *B. dorsalis* and *C. capitata*, though phylogenetically distant, showed convergent sensory responses. Based on volatiles of chicha, we composed a blend and tested this in the field. Although chicha was more attractive, the blend was highly selective to medfly.

This study showed ethological methods for direct application in orchards against *C. capitata*, and an increasing understanding of tephitids evolutionary ecology based on olfactory sensitivity. Therefore, identification of volatile blends and mapping olfactory responses to these can support the development of more effective and species-specific attractants and repellents, with high potential for incorporation into IPM programs.

Keywords: Attractant, repellent, *Ceratitis capitata,* Tephritidae, olfaction, trapping, integrated pest management, fermentation, evolutionary ecology

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Control etológico de la mosca del Mediterráneo *Ceratitis capitata* (Wied.)

Resúmen

La mosca del Mediterráneo *Ceratitis capitata* (Diptera: Tephritidae) es una de las plagas más importantes para el sector frutícola en Bolivia. Aparte del uso de insecticidas, pocos esfuerzos se realizan para el desarrollo de técnicas alternativas, especialmente aquellas basadas en la manipulación del comportamiento a través de la olfacción. Después de evaluaciones en laboratorio y campo, la "chicha", bebida hecha con maíz fermentado, mostró altos niveles de atracción a *C. capitata*, comparado con otros atrayentes locales. Sin embargo gran parte de las capturas fueron otros insectos. Posteriormente, se evaluaron trampas fabricadas con botellas plásticas cebadas con chicha. El prototipo 3 fue comparable en capturas con la trampa comercial Tephritrap. En paralelo, se realizaron experimentos de laboratorio seguidas de pruebas de semi-campo y campo, para evaluar el efecto repelente de aceites esenciales en la atracción, ovoposición e infestación de frutos por *C. capitata*. En laboratorio de demostró que los aceites de *Cymbopogon citratus*, *Tagetes filifolia* y *Schinus molle*, redujeron la atracción hacia el sustrato de ovoposición, mientras que en los experimentos de semi-campo estos suprimieron la ovoposición en duraznos, y en la prueba de campo, gotas de resina sintética biodegradable mezclada con aceite de *S. molle*, redujo la infestación en huertos de durazneros. Se utilizaron la cromatografía de gases asociada con detección antenográfica (GC-EAD) y detección palpigráfica (GC-EPD) para caracterizar los perfiles de volátiles de aceites esenciales (repelentes) y la chicha (atrayente alimenticio) con sus correspondientes respuestas sensoriales. Los tres aceites están dominados por terpenoides y difieren sustancialmente in la clase de compuestos, sus cantidades relativas y el patrón de respuestas antenales/palpales. Por otro lado, comparando las respuestas antenales y palpales a la chicha, tres especies de tephritidos: *C. capitata*, *Zeugodacus cucurbitae*, *Bactrocera dorsalis* y la no relacionada *Drosophila melanogaster*; se sobreponen considerablemente con las respuestas a los volátiles de frutas. Esto lleva a conjeturar que éstos volátiles pudieron servir como "puentes preadaptativos" que ligan a los ancestrales sustratos en descomposición con las frutas en maduración. Sin embargo estas respuestas también divergen con el nicho ecológico: la especialista *Z. cucurbitae* fue más divergente, mientras que las generalistas *B. dorsalis* y *C. capitata*, aunque distantes filogenéticamente, muestran convergencia. Este estudio mostró métodos etológicos de directa aplicación en huertos contra *C. capitata*, y un creciente entendimiento de la ecología evolutiva de los tephritidos basado en la sensibilidad olfativa. Estos esfuerzos de mapeo sensorial hacia la respuesta olfativa a productos fermentados y aceites esenciales, contribuyen al desarrollo de atrayentes y repelentes más efectivos y específicos, con alto potencial para su incorporación en prácticas MIP.

Palabras clave: Atreyente, repelente, *Ceratitis capitata*, Tephritidae, olfacción, trampeo, aceites esenciales, manejo integrado de plagas, ecología evolutiva.

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Dedication

To my beloved family: Noyana and Adhara my daughters, and my wife Eliana, reason of my life. You believe in me, we set up this pathway together, you sacrificed much more than me in this adventure. This achievement had not been possible without your permanent support.

My parents, Margarita and Simón Alberto and my brother Marcos for their encouragement every time throughout my education, professionalization and career until the PhD achievement.

Study nature, love nature, stay close to nature, it will never fail you.
Frank Lloyd Wright

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List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Figueroa Candia I**., Bautista V., Larsson Herrera S., Walter A., Ortuño Castro N., Tasin M. & Dekker T. (2018). The potential of locally sustainable food baits and traps against the Mediterranean fruit fly *Ceratitis capitata* in Bolivia. *Pest Management Science*. doi:10.1002/ps.5286

II **Figueroa Candia I**., Orellana C., Salazar N., Larsson Herrera S., Tasin M. & Dekker T. (2019) A bouquet of plant secondary metabolites reduce attraction of the Mediterranean fruit fly (MANUSCRIPT)

III Larsson Herrera S., **Figueroa Candia I**., Biasazin TD., Tasin M. & Dekker T. (2019). Andean beer links saprophagy to frugivory in Tephritidae. (MANUSCRIPT)

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The contribution of Ilich Alberto Figueroa Candia to the papers included in this thesis was as follow

I Designed and conducted experiments, analysed data and wrote the manuscript together with the co-authors.

II Designed and conducted behavioral experiments in laboratory and semi-field, assisted in field trial, performed all electrophysiological recording of *C. capitata* with the essential oils, analysed data and wrote the manuscript together with the co-authors.

III Designed and conducted field trial with synthetic blend, performed electrophysiological recording of three species of tephritid fruit flies, analysed data and contributed to the writing of the manuscript together with co-authors.

Abbreviations

EAD	Electroantennographic detection
EO	Essential oils
EPD	Electropalpigraphic detection
FID	Flame ionization detector
FTD	Fruit fly per trap per day
GC	Gas chromatography
GC-EAD	Gas chromatography - electroantennographic detection
GC-EPD	Gas chromatography - electropalpigraphic detection
GC-MS	Gas chromatography - mass spectrometry
HIPV	Herbivore induced plant volatiles
IPM	Integrated Pest Management
KI	Kovats retention indices
MS	Mass spectrometer
SIT	Sterile insect technique
SPLAT	Specialized pheromone & lure application technology
SPME	Solid phase micro extraction

1 Introduction

The Mediterranean fruit fly, *Ceratitis capitata* Wiedemann, 1824 (Diptera: Tephritidae) is considered a major pest of fruit production worldwide (Malacrida *et al.*, 2007). Female adults of this species oviposit into ripening fresh fruit, their maggots cause internal damage when fruits are still in the tree. *C. capitata* has the ability of adaptation to several different environments and is highly polyphagous, with a diverse host range including nearly 300 fruits and vegetables, both cropped and wild (Liquido *et al.*, 1991). The species' adaptability and global fruit trade has allowed *C. capitata* to become a hazardous invasive species, with a large destructive capacity that threatens commercial fruit production in every area where it is present (CABI, 2014).

From its centre of origin in east-central Africa, *C. capitata* spread throughout the continent to the south and the Mediterranean basin in the north, including the European side (Gasparich *et al.*, 1997). From this site, the global invasion progressed to Central and South America, Western Australia, the Middle East and the Hawaiian islands (Malacrida, *et al.* 2007). The species is responsible for high expenditures by countries confronted with its invasion such as Mexico, Guatemala, Chile, Peru and some states of USA (California, Florida), which put a lot of public effort to avoid and/or eradicate any presence and prevent for further invasions through extensive monitoring systems (Aluja and Mangan, 2008; CABI, 2014; Enkerlin *et al.*, 2015).

The Mediterranean fruit fly is widespread in Bolivia (CABI, 2014; SENASAG, 2010) The first report of its presence in the southern Bolivian valleys is from the 1940's reporting damage into peach and pears (Mendoza, 1996), suggesting that this invasion originated in Argentina and moved throughout the country until reaching most fruit productive zones in a wide range of regions (SENASAG, 2010). Besides direct damage, *C. capitata* presence makes the country unable to export fresh fruits due to international trade regulations. Fruit growers and some isolated institutions have attempted to develop control strategies in rural productive orchards, including cultural management, mass trapping and chemical application. However, fruit production remains heavily affected in highly fruit productive areas such Cochabamba valleys (Figueroa, 2005). Local strategies for medfly control include the use of home-made traps fabricated from plastic bottles and baited with kitchen fermented ingredients, but their potential for use as mass trapping tool in an integrated management program has not been properly evaluated (SENASAG, 2010)

For an effective control against *C. capitata*, any strategy should start prior to fruit ripening, when females are active in food foraging and host searching (Demirel, 2007). Since host detection is strongly mediated by olfactory cues in tephritids (Yuval and Hendrichs, 2000), a good understanding of how these females behave during this particularly stage, and how this behaviour can be disrupted is important. This thesis focuses largely on odor-mediated behavior of *C. capitata*, and how this can be harnessed in the control of this pest. For this I studied the behavior of adult's females when exposed to natural products, both attractants and plant-based repellents, and scored how this can be employed to trap, reduce and/or suppress the oviposition and consequently reduce fruit damage. I subsequently discuss the possible use of these novel approaches as practical and inexpensive tools that contribute to environmental friendly control of this pest by fruit growers of the Cochabamba region in Bolivia.

2 Background

2.1 Life history of *Ceratitis capitata*

2.1.1 Morphological description and distribution

Adults medfly are small, about 6 to 8 mm long, yellowish in overall color. It has a tinge of brown especially in abdomen, legs and the wing markings (Carroll *et al.* 2002). The thorax surface is convex, of shiny cream to yellow color, and spotted with black blotches. The oval-shaped abdomen is covered with a fine, scattered black bristles. It has two narrow, transverse light coloured bands on the basal half. Females can be recognized by their long ovipositor at the tip of the abdomen, which contains a hard sclerotized organ called *aculeus* (CABI, 2014; Figueroa, 2005; Carroll, 2002), this organ is used to insert eggs through the fruit skin during oviposition (Yuval and Hendrichs, 2000). Male heads bears two long, black bristles with flattened diamond shaped tip, arising between both eyes near the antennae (Carroll *et al.* 2002). (Figure 1B)

Figure 1: (A) Adult female *C. capitata* dorsal view. (Picture by I. Figueroa) (B) Adult male *C. captitata* side view with the black bristles in the head (Picture by Jari Segreto, www.flickr.com)

C. capitata originated in sub-Saharan Africa. Since the 1880's, it has spread throughout the Mediterranean region, southern Europe, the Middle East, Western Australia and eventually the Hawaiian islands. In the western hemisphere, *C. capitata* invasion started in Brazil and Argentina, and then spread throughout most of South American countries (Gasparich, *et al.*1997). From Central America (Panamá, Costa Rica, Nicaragua, Honduras, and Guatemala) *C. capitata* is threatening Mexico and North America, where detections and often outbreaks carried large economical and logistical efforts to block further spreading in those countries (CABI, 2014; Enkerlin *et al*., 2015) (Fig. 2).

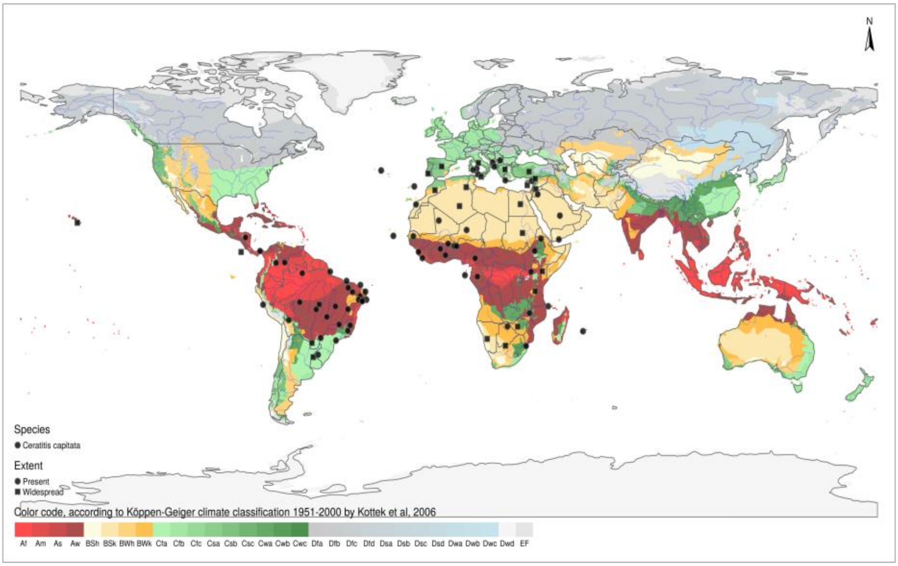

Figure 2: *Ceratitis capitata* distribution map (based in updated data from CABI 2019, and Szyniszewska and Tatem, 2014). Illustration: Sebastian Larsson Herrera

2.1.2 Life cycle

The life cycle of *C. capitata* starts when a gravid female lays eggs inside a ripening fruit. When maggots emerge they start to eat on the fruit flesh (mesocarp and endocarp) causing the characteristic fruit fly damage (Demirel, 2007). Larvae tend to occur close to seeds or pits in the case of stone-fruits, and try to feed mostly in the matured part of the flesh (Yuval and Hendrichs, 2000). When maggots complete their growth, they burrow their way out of the fruit, often creating a visible exit hole and drop on the soil surface. Mature larvae dig into the soil and pupate at a few centimetres depth. Pupae are elliptical and brown colored, and if the environmental conditions are good (high temperature and humidity), adult flies emerge from the puparium and from the soil in 10-20 days (CABI, 2014). In temperate environment with a defined winter or dry season, pupae can remain quiescent over several months until the climatic conditions are favorable (Malacrida, 2007). Adult males show a complex aggregation behavior and form small groups called leks, where they court to potential females (Eberhard, 2000; Yuval and Hendrichs, 2000).

2.1.3 Host range

C. capitata is one of the world most destructive fruit pest, it has a high ability to tolerate cooler climates and is able to spread over a wide range of tropical, temperate, humid or dry ecosystems (CABI, 2014). This flexibility is facilitated by the very large host range in which the species can develop (Szyniszewska and Tatem, 2014). *C. capitata* attacks more than 260 different species of fruits, flowers, vegetables and nuts (Liquido *et al.*, 1991). Usually, thin-skinned, succulent fruits are preferred, such as peach, apricot, guava and mango. Host preferences vary in different regions and some fruits, such as several cucurbit species have been recorded as host only under laboratory conditions and may not be attacked in the field (CABI, 2014). Knowledge of host preference in one region often helps to predict the most likely host to be infested in a newly invaded area, but what may be a preferred host in one particular zone may be a poor host in another (Yuval and Hendrichs, 2000).

2.2 Mating behaviour.

2.2.1 Lekking or aggregation by males

Adult males show a complex aggregation behavior and form small groups called leks, males aggregate on tree foliage where they display behaviors that render them attractive to females (Eberhard, 2000; Yuval and Hendrichs, 2000). The definition of ‘lek’ is a non-resource based male aggregation, visited by females only for copulation (Yuval and Hendrichs, 2000). A sexual mature male starts establishing a territory, usually on the foliage or on the underside of leaves, and releases pheromone by turning up the abdominal tip. He also displays acoustical and visual signals in order to attract females (Demirel, 2007; Shelly, 2000). This activity often attracts other males, which then form a lek, where males interact with each other through displays, short encounters and combats. Although lekking males seem to be more successful in terms of courting and reproduction, there is little evidence of significant difference in mating success between them and a single resting male (Demirel, 2007), however, lekking males were more robust, heavier and with more content of sugar and proteins compared to resting males (Yuval *et al.*, 1998). Pheromone blends released by males are composed of no less than 12 semiochemicals, including methyl-(*E*)-6-nonenoate, ethyl-(*E*)-3-octenoate, (*E*)-2-hexenoic acid and ethyl acetate among others (Siciliano *et al.*, 2014). It is currently still unclear which compounds and/or blends induce female attraction (Benelli, *et al.* 2014). However, the responsible pheromone may be very close to the synthetic formulation of Trimedlure (Shelly, 2000), a broadly used para-pheromone which is very attractive to males, and improves mating success of exposed males (Shelly *et al.*, 1996).

When a female is approaching, she usually walks with the wings in downward position toward the chosen male and faces him, who in response puts his abdomen under his body and begins to vibrate his wings in a perpendicular position of his body. If the female still faces the male, he begins to a second type of wing movement buzzing rapidly forward and backward toward the female. Additionally, the head is moving rhythmically side to side. Finally, once with the female remaining motionless, the male leaps onto the female back and attempts to copulate (Yuval and Hendrichs, 2000).

2.3 Host location and host selection behaviour

Females forage for food immediately after emergence and search for carbohydrates and protein sources, including fruit juices, nectar, yeast from rotten fruits and even bird faeces (Yuval and Hendrichs, 2000). Feeding on proteinaceous substrates is very important for sexual maturation and egg production (Placido-Silva *et al.*, 2005). Virgin females appear to disperse further than males in search for food, which sometimes results in colonizing new locations (Yuval and Hendrichs, 2000). When successful mating occurs, females switch from food foragers to host foragers for oviposition, and start searching for suitable fruits for oviposition (Levinson *et al.*, 1990).

2.4 Olfactory and visual cues in host searching

Once ovaries are matured, females tend to stay on or close to host plants where ripening fruits are available for egg laying. They also can disperse rapidly once fruit become scarce or in response to competition at high fly densities (Hendrichs and Hendrichs, 1990). Major stimuli that mediate orientation in *C. capitata* females searching for oviposition sites include volatiles emitted from ripening fruits, green leaf volatiles and fermenting fruit in the ground surrounding fruit trees (Yuval and Hendrichs, 2000). Visual cues such shape, size and color also play an important role, with spheres being preferred to other shapes and larger to smaller fruits (Katsoyannos, 1989). In spite of preferences that female *C. capitata* display, they are behaviourally plastic during host location and oviposition, which makes this species highly adaptable to several host species and new environments where they are able to colonize new niches and accept poorly suited hosts for oviposition (Yuval and Hendrichs, 2000).

2.4.1 Oviposition behavior

Once a fruit is located and gravid medfly landed, she evaluates the suitability for eggs acceptance based on chemical and physical characteristics like surface structure, fruit maturity, etc. (Katsoyannos, 1989). Upon acceptance she inserts her *aculeus* and injects a clutch of eggs a few millimeters under fruit skin. Correct fruit assessment is critical for the future offspring development, since bigger and close to ripening fruits will give them the possibility to grow with less competitors (intra and interspecific) (Demirel, 2007). It is also known that female *C. capitata* may upon oviposition deposit a oviposition-marking pheromone that averts conspecifics. A gravid female is thus able to detect and discriminate between already infested and uninfested fruit (Yuval and Hendrichs, 2000). As eggs and larvae are well protected in the fruit against control measures, any control strategy design must be aimed at oviposition avoidance (Malacrida *et al.*, 2007).

2.5 Pest management strategies for *C. capitata*

There are many control methods for *C. capitata*: cultural, mechanical, ethological, biological, chemical, and regulatory. All can be included in Integrated Pest Management (IPM) programs against Medfly:

2.5.1 Cultural control

Cultural control refers to activities within the field or fruit orchard, and includes tillage, weeding, pruning, damaged fruit removing and disposal, irrigation, fertilization, etc. It is developed and recommended by research centres and fruit growers organizations (SENASAG, 2010), but has the disadvantage of high labour intensity. Of all cultural practices, removal of damaged fruit and adequate disposal of decomposing material seems to be the most effective. The activity alone may disrupt the fruit fly cycle and then reduce populations and avoid further sources of infestation (Adell *et al.* 2013). Removing damaged fruits also contribute to population decline by eliminating food sources for adult male and females. Fruit are either burned, or buried with the addition of ashes or hydrated lime (calcium oxide) spreading over buried fruits (SENASAG, 2010). An alternative to burying is fruits chopping, grinding and spreading the remains for desiccation and/or composting (Adell *et al.* 2013).

2.5.2 Regulatory control

Regulatory control is related to any mandatory actions regarding fruit production, trade and transportation. These regulations can be agreed upon among growers of certain productive areas in small scale, or a full-country laws promulgated by sanitary authorities that affect entire countries and their entrance ports (Enkerlin *et al.* 2015). Regulatory control may include: (i) mandatory application of control strategies in orchards, house backyards, gardens, and public areas where fruit trees are present, (ii) restriction to fruit movement from one region to another, including fresh fruit trading and consumption, (iii) establishment of quarantine zones where a fruit fly outbreak is detected. All these regulations are designed to prevent further invasions and outbreaks by creating legal barriers (Suckling *et al.* 2014). Mandatory regulation is very important in regions and countries where large control and eradication programs are being executed. Regulatory control allows to country authorities to achieve successful results when all stakeholders are committed by law in the fruit-fly control program (Enkerlin *et al.*, 2015).

2.5.3 Sterile Insect Technique

Sterile insect technique (SIT) is also known as ‘autocide control’, because of the use of mass reared sterile insects of the same species to suppress population growth. SIT against medfly population is widely used despite its cost and long-term engagement, and has demonstrated to be a successful way to control and eradicate medfly populations, and thereby lift quarantine measures from certain areas. This technique is also considered the most non-disruptive pest control method, as it is species-specific and without the use of toxic products or introduction of new genetic material into existing populations of target insects (Hendrichs, *et al.* 2002). SIT requires advanced facilities and equipment in order to produce very large quantities (millions of individuals) of sterile *C. capitata* males, by irradiation of pupae with gamma rays and other sources of radiation. Irradiated pupae are inundatively released to induce mating of females with infertile males instead of wild males and cause population suppression (Enkerlin *et al.*, 2015, Suckling, *et al.* 2014) Key to success of this technique is an overabundance of sterile males related to the wild ones, and minimizing the chance of females to mate with fertile, wild males (Hendrichs, *et al.* 2002).

Currently this type of control is used in countries which face medfly invasion from border countries (México, Chile, USA-California, Florida) and/or that wish to eradicate already established populations in some of its regions (Guatemala, Argentina, Peru, Australia, USA-Hawaii) (CABI, 2014). This means a huge expenditure, but the gains may be worth many times the investment, not only because of the increased production, but particularly of the access of premium price markets through export, once the region or even a whole country is declared medfly-free (Enkerlin, *et al.* 2015)

2.5.4 Ethological control

Ethological control is related to the behavior manipulation of target pest, in order to minimize its reproduction and/or targeting the plant/crop. This can be done using visual approaches or by semiochemicals, including insect-pheromones, food attractants, repellents, etc. (Aluja and Rull, 2009) Although there is not yet a well-developed synthetic sex pheromone for *C. capitata*, a synthetic alternative, trimedlure, exhibits pheromone-like attraction of males. Trimedlure was discovered in the early 20th century and is frequently used as part of monitoring and control *C. capitata* (Shelly *et al.*, 1996; Díaz-Fleischer *et al.*, 2014). Ethological control using food attractants such as proteinaceous-based baits is commonly employed in monitoring and mass trapping programs (Epsky, et al. 2014). Some strategies, also use attractants combined with insecticides in order to lure-and-kill the more adults possible (Suckling, *et al.* 2014). Mass trapping continuous to be heavily commercially researched, and includes besides effective lures, a search for a good trap design (Navarro-Llopis and Vacas, 2014). While mass trapping with male lures and food attractants has been used for many years throughout the world, little research has been done on using repellents, deterrents or the combination of them in systems like push-pull (Aluja and Rull, 2009). The addition of insecticides to food attractants has shown to be effective in population suppression of tephritid fruit flies (Epsky, et al. 2014). This approach is generally named attract-and-kill. The principle behind this strategy is luring adult flies, primary females, for ingesting the attractant and then die by intoxication. Historically, proteinaceous food attractants were combined with organophosphate toxicants (Revis, *et al.* 2004), but more recently with Spinosad, a mixture of spinosyns A and D, both metabolites of the soil microorganism *Saccharopolyspora spinosa* (Dow AgroScience). This product has high toxicity for insects at low concentration, and are used widely in pest control for several species including mosquitoes and tephritid fruit flies (Stark, *et al.* 2004)

Spinosad-based toxic lure, mainly traded as ‘GF-120’ is used as an important tool into integrated programs for fruit fly control, as a primary suppressor of new outbreaks in areas free of *C. capitata* like Mexico, California, Argentina, Peru and Chile (Enkerlin, *et al.* 2015). One disadvantage of GF-120 is its short field life. In studies for control of the Melon Fly *Bactrocera cucurbitae* in Hawaii, a difference of 2 hours after application resulted in 11 times less attractive and 50% ineffective when exposed to rain (Revis, *et al.* 2004).

Another approach of the attract-and-kill principle is the mixing of male attractants with spinosad. This technique was evaluated with methyl eugenol, a well-known male lure of the genus *Bactrocera* (Tan *et al.*, 2014). A combination of methyl eugenol together with spinosad into a base matrix formulation of inert materials called specialized pheromone and lure application technology (SPLAT) (Vargas, *et al.* 2009). This A&K formulation gave prolonged control due to the controlled release rate of the attractant and resistance of the formulation against washing out, reportedly providing control for up to 4 weeks gives many possibilities of application due to the wide range of viscosity that are available. This product has shown particularly good perspectives in area-wide of control of particularly *Bactrocera dorsalis* in Hawaii (Vargas, *et al.* 2009).

2.6 The stimulo-deterrent diversion strategy (*push-pull*)

Control of a target pest can be more effective when repellents are combined with attractants in the same environment. This combinatorial strategy is known as push-pull and is related to the behavioral manipulation of insect pest, making the crop unattractive (push), while luring them to attractive sources (pull) using non-toxic components such repellents, attractants, attractive plants, crop-traps, etc. (Cook *et al.*, 2007, ; Khan *et al.*, 2008, Deletre *et al.*, 2016) When push-pull is used with natural stimulus sources such as plants or crops, for instance repellent plants and trap-crops, it may increase natural enemies’ activity and soil fauna (Midega, *et al.* 2009), the result is a decreasing pest population and a reduction in damage to the main crop.

This strategy has been developed successfully in protecting maize crop from stemborer moths (*Busseola fusca*, *Chilo partellus* and others) in Western Africa (Khan, *et al.* 2008). Traditionally, it consists of a repellent intercrop *Desmodium* crops in maize fields to repel the pest in maize fields, and a trap crop (an unsuitable, but attractive plant for the pest) in the field’s perimeter to divert the pest to an alternative host (Khan and Pickett, 2004). From there, several other control systems have been being developed using similar principles (Cook, *et al.* 2007).

The push pull strategy against a fruit flies species was researched in olive orchards against the olive fruit fly *Bactrocera oleae* using kaolin as oviposition deterrent and bait traps at the same time in olive orchards from Sardinia, with a comparable efficiency of orchards treated with pesticides (Delrio *et al.* 2010). Some other experiments with other species of fruit flies such as *Bactrocera* and *Anastrepha* spp. included the use of extracts of fly-faeces as oviposition deterrent combined with traps baited with a food attractant (Aluja and Rull, 2009).

A good understanding of the pest chemical ecology and its interaction with the environment is essential to develop any ethological strategy to control tephritids (Aluja and Rull, 2009). For instance, in a study of *Rhagoletis* fruit fly species, a lower risk of oviposition was achieved when an efficient food attractant was combined with an early application of host marking pheromones (Sarles, *et al.* 2015). *C. capitata* faeces also contain oviposition deterrent and may be used to reduce infestation. Application of concentrated Medfly faeces on coffee bushes, reduced infestation up to 80% in treated coffee berries and 56% in adjacent non-treated bushes, showing a spatial repellence extending to nearby plants. (Arredondo and Diaz-Fleischer, 2006). Fruit-fly semiochemicals show good potential in integrated management programs. However, usually one approach is not enough to achieve optimal results in pest suppression, i.e. trapping with only male lures. Therefore, a combination of more than one approach should be considered in ethological fruit fly control (Silva, *et al.* 2012)

A successful example of this is the control of cucurbit infestation by tephritids, primary *Zeugodacus cucurbitae*, which was achieved on the island of La Réunion. This experience included the use and/or combination of trap crops, attractants trapping, fruit sanitation, augmentoria and insecticide baits (Deguine, *et al.* 2015). Good results were achieved due to some fundamental factors: (i) a well-known pest status in the island's agroecology based on several years of research, and a good knowledge of the life cycle, seasonal occurrence, adults ecology and behavior regarding mating and oviposition. This gave researchers a good picture over where and when apply strategies. (ii) A well-coordinated action involving all stakeholders in the cucurbit production sector of La Réunion. This coordination, allowed growers to get introduced and adopt most of the strategies. The strategy was effective in reducing the pest population and strongly reduced pesticide application (Deguine, *et al.* 2015).

2.7 Chemical ecology in medfly related to attraction and repellence

2.7.1 Sensory organs related to olfaction

Chemical signals play an important role in any ecosystem, they influence behavior, physiology and ecology of insects in various ways, including plant-insect interactions, insect-insect interaction and insects-microbe interaction (Schoonhoven *et al.*, 2005). The sense of smell is key for most of flying insects, and they have mostly a highly developed olfactory sensory system (Hansson and Stensmyr, 2011). Chemical signals that denote behavior response in insects, are usually related to food searching, sexual communication, host location, host suitability assessment, etc. (Yuval and Hendrichs, 2000; Benelli *et al.*, 2014)

Insect primarily sense odors using the antennae and maxillary palp. Both organs are covered by different types of sensilla with pores through which odorants enter the sensillar lymph. Typically, each sensillum contains two or three olfactory sensory neurons (OSN) each usually expressing a single type of odorant receptor (OR), which responds to a limited range of chemical compounds. In the lymph, odorant binding proteins (OBP) bind to odorants that chaperone odours to the dendrite that expresses olfactory receptors (Leal, 2013, Hansson & Stensmyr, 2011). Olfactory studies often focuses on antennal responses, whereas little attention has been given to the characterization of maxillary palpae. The functional role of the palpae may be related to taste enhancement in *D. melanogaster* due to an observed overlap on the response to odor volatiles as well as location of maxillary palp closer to proboscis (Dweck et al., 2016). There is evidence that both antenna and palp of *Bactrocera dorsalis* are important in detecting methyl eugenol in which palpae have shown to be important on short range detection (Chieng *et al.,* 2018)

2.7.2 Food attractants

A close definition of “attractant”, may be a substance or blend of substances that exerts in an exposed insect a behavior of movement toward the source of such a substance. (White, *et al.* 2014). Food attractants are those that are a feeding source or mimic one. Early control strategies of tephritid trapping used products such fermenting sugar baits, yeast and by-products of beer industry such as brewers waste and related, modified protein by hydrolysis (hydrolysed proteins) and ammonia solutions, among others (Epsky et al., 2014). Synthetic products and protein-based lures have been used as attractants in monitoring and control programs (CABI, 2014). These attractants include hydrolysed protein (from soybean, maize, whey and others), and torula yeast used as components in single or blended baits, for attraction of both males and females in mass trapping programs, or combined with insecticides into attract-and-kill systems (Benelli *et al.*, 2014). Proteinaceous lures are highly attractive for females searching for food and also oviposition hosts (Placido-Silva *et al.*, 2005).

Efforts to identify active compounds from those food attractants, have pointed to ammonia (the primary product of protein hydrolysis) as a key attractant (Mazor, 2009). Other compounds were also used to enhance attractiveness, like combined formulations of ammonium acetate and putrescine (Heath, *et al.* 1997). Most of compounds related to food attraction for tephritid fruit flies are available as commercial products for monitoring and mass trapping including "Buminal ®", TYB, BioLure, AFF lure and others (Epsky et al., 2014). Although food attractant are a powerful tool for capturing target fruit flies, one disadvantage is the high level of non-target captured insects including beneficial like bees, wasps and lacewing.

The use of food attractants in trapping involves a great variety of trap designs (Tan, *et al.* 2014). The most widely used trap for liquid food attractant is the "McPhail" trap with a beneath funnel principle, in which the attracted fly is lured through an inverted funnel of its base inside trap, into the top container. The transparent upper part of the trap induces positive phototaxis and prevents flies to exit through the bottom opening. Eventually the captured fly will die mainly by drowning in the liquid bait (Navarro-Llopis and Vacas, 2014). Some variants of this trap were developed, and eventually the "Tephritrap" which is considered an effective trap in catching tephritid fruit with liquid baits. The design include the same inverted funnel principle with the addition of four small circular windows in four directions in the middle part of the trap, reinforced with tubes (funnel like) inside (Miranda, *et al.* 2001). These windows allow better release of attractive volatiles and also provide multiple entry points for flies.

2.7.3 Male lures

Another kind of attractant that is frequently used in monitoring and mass trapping is the aforementioned male lures. Ingestion of these compounds (naturally or synthetic) by male flies may induce synthesis of male aggregation pheromones (Shelly et al., 1996; Tan, *et al.* 2014), which is important for the chemical communication among males for lekking and for successful mating (Yuval and Hendrichs, 2000). The most used male lures are methyl eugenol, raspberry ketone and cue-lure, which attract males of most of *Bactrocera* and *Dacus* species (Tan, *et al.* 2014).

Trimedlure and Ceralure are synthetic male lures that attract *Ceratitis* species including the Mediterranean fruit fly *C. capitata*. (Avery *et al.*, 1994). Trimedlure (t-Butyl-2-methyl-4-chlorocyclohexanecarboxylate, various manufacturers) is a synthetic compound, which has been adopted as chief male lure for *C. capitata* in detection and surveillance programs (Jang el al. 2001). Trimedlure has a powerful effect in attracting *C. capitata* males. It was used in liquid presentation, or included in releasing solid dispensers like rubber septa and polymer plugs. Field life may vary from 4 to 30 weeks depending on the concentration and dispenser quality (Tan, *et al.* 2014). Trimedlure and other male lures are used mainly in sticky traps (like Jackson traps) which are prisma-shaped traps made of plastic or carton. The inside of these trap contain a sticky surface that maintains its feature in exposed environments (Navarro-Llopis and Vacas, 2014).

2.7.4 Repellence

Nerio, *et al.* (2010) defined a repellent as a substance that act locally or at a distance, deterring an arthropod from flying to, landing on or biting human or animal skin (or surface in general). Different from toxic plant-based pesticides, whose primary goal is killing a target insect population, repellents act generally through generating a spatial barrier avoiding the target insect from coming toward a protected surface, such a human skin, spaced place or even crop plots (Regnault-Roger, *et al.* 2012). This feature of spatial repellence, differs from the so called “deterrent”, which is a compound whose presence causes avoidance or interruption of certain behavior upon direct contact with the compound (Arredondo and Diaz-Fleischer, 2006). It is reported that female fruit flies release substances together with egg depositions when infesting fruits (Yuval and Hendrichs, 2000). Some of these excreted compounds during oviposition also generate rejection for con-specific competitors such other gravid females. This kind of rejection to an already infested host is known as “oviposition deterrent”, and is especially important when suitable resources are scarce. Oviposition deterrent in fruit flies may come not only from released compounds during oviposition but also from faeces. (White, *et al.* 2014)

Emitted odours by non-host plants, may exert also repellence in those insects searching for suitable hosts (Khan, *et al.* 2008). For instance, when a gravid fruit fly is searching for hosts, she will fly toward primarily to those odor sources exerting ripening fruits instead of those with non-ripe fruits and/or non-suitable host (fruits with toxic compounds, antifeedants substances, etc.) (Demirel, 2007). In other cases, herbivore induced plant volatiles (HIPV) emitted by the host-plant, can act as repellent for the adult stage of the herbivore. This is evident in the cotton moth *Spodoptera litoralis*, when host-plant derived HIPV interfere the sensory system in the search and host location activity (Hatano, *et al.* 2015)

Botanical products can have compounds with potential repellence against pestiferous insects (Moore, *et al.* 2014). Lemon grass oil (*Cymbopogon citratus*) induced strong responses in an olfactory receptor of the stable fly *Stomoxys calcitrans*, and repelled flies under laboratory conditions (Baldacchino, *et al.* 2013). Lemon grass oil and its main active components geranial and neral were tested for repellence activity against different species of disease-transmitting mosquitoes (Deng, 2014) and the common house fly (*Musca domestica*) (Kumar, et al 2011; Morey 2012), making this particular plant species, interesting for prospective research in dipteran repellence. There is potential of certain botanical product, such as essential oils to be used as spatial repellents against herbivorous insects in crop protection (Isman, 2006). However, most research effort in repellence have focused on blood sucking insects, and frequently in relation to public and livestock health (Nerio, *et al.* 2010). Research on natural essential oils for anti-insect purposes is gaining interest in an attempt to generate alternatives to synthetic pesticides (Isman, 2006).

Although most of chemical ecology techniques for semiochemical's research are well developed, they are used mainly for attractant semiochemicals (sex pheromones, para-pheromones, attractant volatiles and so on). Whether such techniques are suitable for assessment of repellence is not known yet, inasmuch as the mode of action of repellents is largely obscure (Deletre, *et al.* 2016; Moore, *et al.* 2014). Nevertheless, a general approach to detect and evaluate repellent compounds follows: (i) attention of plant-based product to be tested. (ii) General screening by behavioral bioassays (iii) Chromatography techniques for key compounds identification, such as GC-MS (Gas chromatography and mass spectrometry) and GC-EAD (Gas Chromatography linked Electro-Antennographic detection). (iii) Compound assessment by behavioral bioassay programs (Epsky, et al. 2014). This technique was useful to identify some mosquito repellent compounds from fresh crushed plants of *Ocimun forskolei*, which is used as repellent plant in some African countries (Dekker *et al.* 2011). The disadvantage of GC-EAD is evident, because a sensory response detected by the technique does not necessarily relate to repellence. Some compounds causing repulsion might block or suppress the sensory system, and may thus not induce a sensory response.

Mosquito repellents are the most researched products due to their high effectiveness in preventing biting and blood-borne disease transmission (Deng, *et al.* 2014). In-vitro techniques were developed as behavioral bioassays in order to evaluate repellence activity instead of using human voluntaries for biting. Some include using landing arenas with and without repellence treatment, adaptation of Y-tube olfactometers, membranes, etc. Key in any assessment is the use of a proper attractant or stimuli in every test, and the outcome should be the successful blocking of such attractiveness by the repellent (Deng, *et al.* 2014). Other techniques for the analysis of behavior include the video tracking of treated and untreated arenas and the subsequent time and/or surface of subjects staying in one or other arena. These methodology were used to determine the repellence of lemon grass oil (*C. citratus*) against the stable fly *Stomoxys calcitrans* (Baldacchino, *et al.* 2013).

3 Aim and objectives

The aim of this thesis is to develop ethological control components against the Mediterranean fruit fly *Ceratitis capitata,* based on olfactory studies of both attractants (food baits) and repellents (plant extracts), to use in integrated pest management in high value peach production in Cochabamba, Bolivia. In order to achieve this aim, the following objectives were considered:

1. **To assess the effectiveness and specificity of proteinaceous-based lure and trap designs in trapping adult medflies into peach orchards.**

Proteinaceous and fermented products have historically been used for fruit fly attraction and trapping, but sometimes they are expensive and not locally available for fruit growers. In this objective local baits are tested and complemented with the evaluation of traps designed with waste plastic bottles, in order to develop a practical and inexpensive approach for fruit fly monitoring and mass trapping, using local available resources.

2. **To evaluate the effectiveness of plant-based extracts in reducing attraction and fruit infestation of *C. capitata*.**

Plant based extracts and essential oils were primarily used for their toxic features against agricultural pest, little is known about their effect as spatial repellents to tephritids in general and the medfly in particular. Therefore, behavioral evaluation of aromatic plant extracts and essential oils are conducted from laboratory to the field, including the exploration of adult medfly physiological response to those essential oils that exert oviposition and/or feeding reduction.

3. **To explore the similarities and dissimilarities in olfaction response by tephritid species including medfly to the food attractant chicha, in order to better understand their evolutionary ecology.**

Since chicha, a fermented product, is highly attractive to *C. capitata,* depth studies on the volatiles that comprised its headspeces and their response to flies’ sensory organs are performed. This evaluation uses three tephritid species and the non-related *D. melanogaster*, allowing to identify those key compounds from the chicha headspace that give active response to antennae and palpae, and also check the similarities and dissimilarities in olfactory response in order to deconstruct their evolutionary sensory ecology.

4 Methods

4.1.1 Tephritids flies

Start-up material for our *C. capitata* rearing was obtained from IAEA (courtesy of Sohel Ahmed and Carlos Caceres, International Atomic Energy Agency, Austria). Two colonies were established with similar standardized rearing methodology in both Bolivia (laboratory of Entomology, UMSS, Cochabamba) and Sweden (SLU-Alnarp). Adults were kept in bugdorm™ cages (325x325x325 mm) with access to both water and a sugar-yeast dry diet. After mating, females oviposited through the mesh of the cage and eggs dropped into trays filled with water. Collected eggs hatched in a wheat-based larval media (Tanaka *et al*., 1969). Rearing and behavioural tests were done in climatic controlled environments (25-27ºC, 60% – 70% R.H and L:D 12:12). Other Tephritidae species used in electrophysiological studies (*Bactrocera dorsalis* and *Zeugodacus cucurbitae,* manuscript III) also originated from IAEA and reared in bugdorm™ cages. Mature *B. dorsalis* flies were provided with an artificial ovipositing/larval media based on carrot power (Ekesi *et al*., 2007) and pumpkin (natural diet) for *Z. cucurbitae*. *D. melanogaster,* Dalby strain (Ruebenbauer *et al*., 2008), was reared on a standard sugar-yeast-cornmeal diet (Becher *et al*., 2010).

For evaluation of attractants in the laboratory (paper I), five days old males and females were used. For assessment of repellence in the T-maze, 6-choice olfactometer and semi-field trials (manuscript II), 8-10 d mated females were used. For electrophysiology tests (manuscripts II and III), *C. capita* females of 5-8 d after emergence were used, and *B. dorsalis* and *Z. cucurbitae* females of 10-14 d after emergence were used, whereas *D. melanogaster* females of 3 d old were used.

4.2 Attractant and repellent products

Chicha, corn-based fermented beverage: This product has a long tradition in the making process and consumption by people in Bolivian rural areas. It is relatively inexpensive and available everywhere in fruit productive zones. This product were evaluated together with others available in the local Bolivian marked. For the laboratory experiments in Sweden, chicha purchase and storage at 4ºC were done in order to preserve its characteristic during transportation and experiment performance.

Plant extracts and Essential oils (EO): 9 extracts were obtained by steam distillation from different plant (see full description in paper II). Then, after first laboratory and semi-filed screaming, three EO obtained from the ‘peruvian peper tree’ *Schinus molle*, the lemon grass *Cymbopogon citratus* and the wild anise *Tagetes filifolia*, were used for spatial repellence evaluation, electrophysiology and chemical analyses. For the subsequent experiment an analysis, new extracts were produced and immediately stored at -18ºC before any new test.

4.3 Olfactometry

In order to perform behavioral experiments in the laboratory, several olfactometry approaches were developed and tested for tephritid fruit flies, primarily adults *C. capitata*. For a repellence evaluation of plant extracts, a T-maze olfactometer was designed, tested and performed. Whereas for the evaluation of attraction of proteinaceous products, as well as single compounds, a two-choice olfactometer without air-flow was designed. Finally, a 6-choice olfactometer was designed, developed and tested with several species of fruit fly including *C. capitata* and then tested not only for attraction assessment, but also for evaluation of repellence of essential oils.

4.3.1 T-maze

The design of the T-maze olfactometer, used for repellence testing, is described in manuscript II. In this setup the position of individual female medflies in the two arms of the setup was recorded during the 10 min experimental time. Standard wheat-based larval media was used as attractive stimuli in both arms of the main tube, whereas only one arm was used with the plant-extract odour as test side. An air flow (1 L/min) was pulled via the release tube through both arms to the T-maze (Fig. 3A)

4.3.2 Two-choice olfactometer with "mini-McPhail" traps

This olfactometer was designed to use the positive phototaxis and negative geotaxis of adult medflies. In addition, the setup tests a group of flies instead of single insects, such as in Y-tube approaches. Flies were released in an acrylic box arena (50 x 30 x 20 cm) fitted with two fly-traps made of a plastic container with inverted funnel in the bottom, resembling the commercial 'McPhail' trap. The box had an opening on one side fitted with a sleeve for fly release. One of the traps was baited with the testing product (proteinaceous attractant), whereas the other was blank (Fig. 3B). This setup is described and used in paper I prior to the field trial.

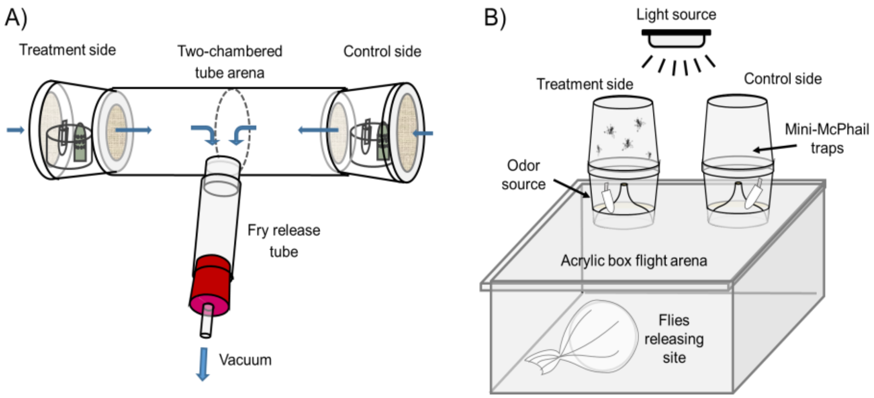

Figure 3: Schematic drawings of (A) T-maze olfactometer consisting of acrylic tubes and capped with fine mesh to prevent flies from escaping. Arrows indicate the direction of the airflow. (B) Two-choice olfactometer with "mini-McPhail traps. Control and treatment sides were swapped between experimental run. Insects were released through a sleeve, seen in the front.

4.3.3 Multi-choice olfactometer

The tephri-group in SLU-Alnarp (www.tephri.org) co-designed and developed a novel 6-choice arena for behavioral assays with adult Tephritidae. This setup allows to competitively screen attractive compounds against each other, as well as test compounds for spatial repellence products (such as essential oils) against each other together with a known attractant. This setup was assembled at UMSS (Bolivia) and at SLU (Sweden). The olfactometer was first described in Biazasin *et al.* (2018) where it was used for attraction evaluation in tephritids to odor eluting from whole fruits in comparison with synthetic mixtures. Manuscript II of the present thesis describes a modification of the original setup that was used for evaluation of synthetic compounds as well as repellence of essential oils against female *C. capitata* (Figure 4). Whereas the modification is more restrictive in the size of the odor sources that can be tested, the airflows are more easily controlled.

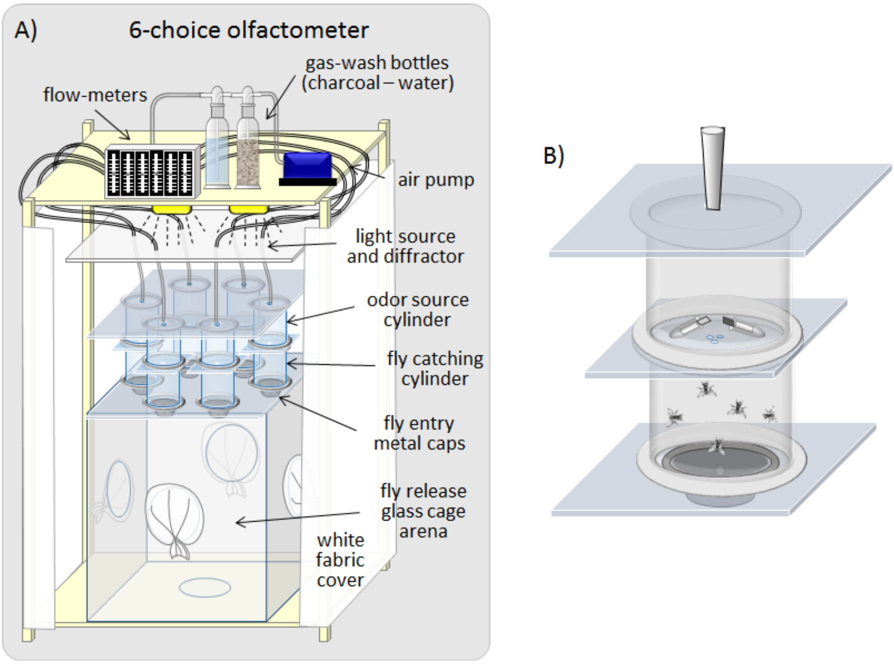

Figure 4: (A) A schematic drawing of the 6-choice olfactometer setup, used for the evaluation of repellence of essential oils against *C. capitata*. (B) Close-up view of a choice chamber: Odor source cylinder (upper, containing eppendorfs with testing products or compounds) and fly catching cylinder (lower cylinder). Illustration: Ilich Figueroa.

4.4 Semi-field and field assays

4.4.1 The agro-ecological region of Valle Alto, Cochabamba,

“Valle Alto” is an inter-Andean valley located southwest of Cochabamba city in the geographic center of Bolivia. This place is characterized by its higher altitude, between 2600 and 2800 m.a.s.l (meters above sea level). Although its latitude (17º 35’ south) places is in the tropics, its altitude makes it Mediterranean in climate and useful for production of fruits typical for temperate regions such as peach, apples, pears and even grapes (Cárdenas, 2009). However, all cultivated varieties need low accumulation of cold hours, since winter is less cold and long than Mediterranean locations (Mendoza García, 2009). Ecologically, this region belongs to the bioclimatic province “Tucumano-

Boliviano dry forest", with a Mediterranean climate, a humid summer and a dry winter with an average precipitation of 450 mm, mainly concentrated between November and March (Navarro & Maldonado, 2002). There is a long tradition of peach production and a high diversity of local varieties. Main constraints for peach productions in "Valle Alto" are lack of irrigation water, small orchard plots (average of one hectare and or 500 trees per farmer) and other climatic factors, such as spring frost or hails (Cárdenas, 2009; Mendoza García, 2009).

4.4.2 Trapping trials

The first trap trials was described in paper I, where the commercial trap "tephritrap" (Pherobank, The Netherlands) was used to test four proteinaceous attractants in several peach orchards of the Valle Alto and Valle Bajo regions in Cochabamba, Bolivia. The tests with trap prototype described in the second part of the paper was performed using six design of traps with plastic bottles (waste material), these prototypes were tested against the tephritrap. Trapping trials described in manuscript III were also used for the evaluation of a synthetic blend and compared using the tephritrap. In all trials, traps were deployed into peach orchards by hanging them at middle height in selected trees. Evaluation was performed weekly by collecting and preserving all capture insects in 70% ethanol. Trap catches were sorted in male and female medfly, and non-target insects which were separated in beneficials (honey bees, predators like lacewings, hoverflies, paper wasps and hymenopteran parasitoids) and others. Weekly trapping evaluation was performed during the fruit season of peaches (March to June) when the medfly population rises and peaks.

4.4.3 Repellent application in semi-field and field:

Semi-field and field bioassays for repellent testing are described in manuscript II: semifield test were carried out using fabric cages (80x80x120 cm) with fruit bags hung at different levels. The upper bag was baited with the product for repellence (essential oil from four species: *Cymbopogon citratus, Schinus molle, Tagetes filifolia* and *Citrus paradisii*). Adult females were then released into the cages and allowed to oviposit by 24 hours. After this time, fruits were removed and kept in plastic containers at room temperature for larval development. The number of larvae and pupae present in exposed fruit was recorded after 10-12 days.

The field trial was carried out using the essential oil of the Peruvian pepper tree, *S. molle* (Anacardiaceae), which appeared one of the most attraction- and oviposition-reducing essential oil in laboratory and semi-field trials. The *S.*

molle essential oil was embedded into 80% SPLAT (Specialized Pheromone and Lure Application Technology). SPLAT-Molle dollops were located in every flank of a tree covering the four cardinal directions, placed at the interior side of the tree in the most extreme part of a branch. Seven days after repellent deployment, fruits located in the half facing the center of the quadrant (inner half) and those outer half were collected and kept separately for fruit fly larval presence evaluation through fruit cutting off (see full description in manuscript II).

4.5 Electrophysiology and chemical analyses

4.5.1 Headspace sampling and chemical analysis

Volatiles constituting the headspace of food attractant chicha and essential oils were sampled using SPME (solid phase microextraction) fibres (coated with DVB/CAR/PDMS). Headspace was collected from samples placed in a 20 ml glass tube fitted with a PTFE-lined septum for 10 min. For chicha SPME headspace sampling, one ml of chicha was used (paper I and manuscript III), whereas for sampling the headspace of repellents, 10µl of pure essential oil on a piece of filter paper was used. In the case of *S. molle*, 10µl of a dilution of 10^{-2} pure oil was used (manuscript II). The adsorbed volatiles collected by the SPME fiber were then thermally desorbed in the inlet of a gas chromatography coupled mass spectrometry (GC-MS) for chemical identification, or the Gas chromatography coupled electroantennogram detection (GC-EAD) for antennal or palpal detection. Active peaks were identified by matching the spectra with that of three reference libraries: Alnarp1609, NIST14 and Wiley275, Kovats retention indices (KI), and synthetic compounds to confirm the primary identification.

4.5.2 Electrophysiology analysis and olfactome database

GC-EAD allows to deconstruct a complex blend in the components and assess which of these induce an olfactory response in the insect's antennae. In this study, this approach was used in both, antennae (EAD) and maxillary palps (EPD), the latter rarely performed before. Electrophysiological recordings on the tip of antenna and palpae were performed to assess which compounds in the headspace of essential oils induced electrophysiological responses in sensory organs (manuscript II), whereas recordings on distal and medio-central parts of the antenna and palpae, were performed to identify volatiles from chicha that may be responsible for its attractiveness to tephritid fruit flies and *Drosophila melanogaster* (manuscript III, Fig. 5).

Recording results allowed to create databases over the compounds that are detected either by antennae and palpae in the tephritid species used in the studies (*C. capitata*, *B. dorsalis*, *Z. cucurbitae*) and correlate these responses of the non-related fly *D. melanogaster*. The data was appended to a database of antennal active compounds from fruit volatiles (Biasazin *et al*., 2018).

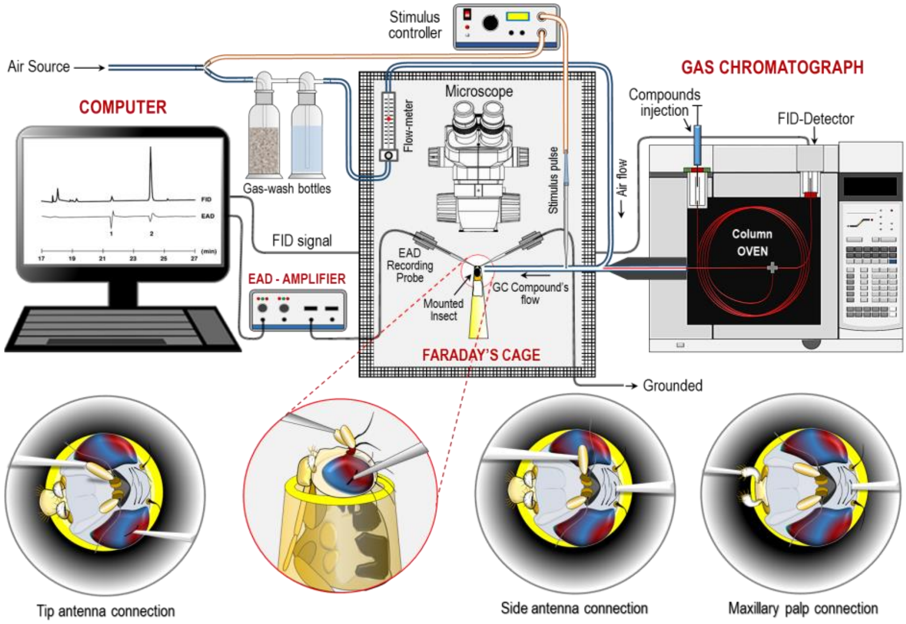

Figure 5. Detailed schematic drawing of the electro-antennogram detection coupled with gas chromatography (GC-EAD) setup in tephritid fruit flies. Close-ups show the mounted fly with the extruded head out of a pipette tip, and the connection detail made for tip antenna, side antenna (EAD) and maxillary palp (EPD). Illustration: Ilich Figueroa.

5 Summary of results

5.1 Part 1: Locally available system for medfly trapping

Chicha, a local fermented corn-based beverage is attractive to medfly

Field trials evaluating the attractiveness of baker's yeast and chicha compared to two commercial attractants (hydrolyzed protein and torula yeast) show that the first two attracted more medfly than the commercial lures, although substantially less specific, catching almost equally many non-target insects (Fig. 6A).

Inexpensive fruit-fly traps for trapping flies with chicha

Since chicha functions as a good attractant, a field trial was performed to test bottle-designed traps using waste materials (PET plastic bottles), with chicha as bait. Trap design should include some principles for better medfly catching, including: lure protection from entering insects, a small entry fitted for tephritid flies, avoiding escapes of captures, and easiness to manufacture. Following these, six prototypes were designed and tested in the field (Fig. 6D). One design, called "T-trap", was equally effective in medfly captures with the commercial tephritrap (Fig. 6B). In addition, it caught fewer beneficial insects (Fig. 6C) (Manuscript I). The T-trap consisted of two plastic PET bottles (cleaned and free of labels): a base bottle contain the bait ("chicha") in the bottom, it has 6 equidistant holes (0.7 mm) in the upper third of the bottle. A second bottle, placed horizontally on top of the first bottle captures insects that move upward using the positive phototaxis and negative geotaxis, which is common in flying insects. They die by suffocation, dehydration and/or insolation (manuscript I).

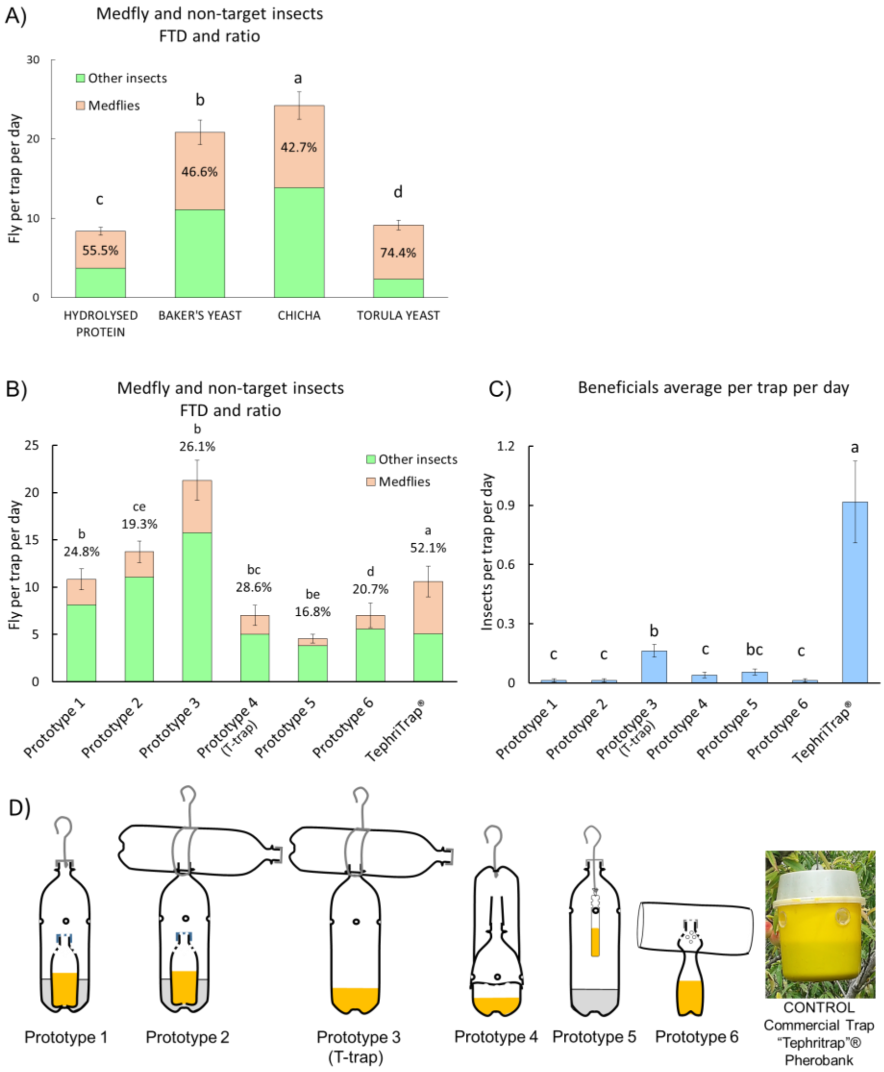

Figure 6. (A) Average *C. capitata* captures per trap and day are displayed for each treatment, with standard error bars. Different letters indicate significant changes between treatments. (B) Average FDT *C. capitata* captures using chicha as bait in different traps, plotted with standard error bars. Letters indicates significant differences between trap types in a GLMM model with a subsequent post-hoc test. (C) Total beneficial insects caught per day and trap prototype. Letters indicates differences in number of captures between the trap types. (D) Schematic drawing of the six different bottle-trap prototypes and the commercial trap “Tephrtrap®” (Pherobank) used as positive control. Figure modified from Figueroa Candia *et al.,* 2018.

5.2 Part II: Plant-based repellent against medfly: From laboratory to the field

Three plant-extracts showed repellence in the laboratory

In a T-maze setup the headspace of nine candidate botanical extracts and essential oils were tested for their repellence activity by adding them to the effluent of oviposition and larval medium odor and tested against media odor alone. Odor from *S. molle*, *C. citratus*, *T. filifolia* or *C. paradisii* extract reduced time spent in that arm compared to the control arm (Fig. 7A). We subsequently tested if essential oils also reduced attraction to food odor, chicha. In a 6-choice olfactometer experiment, indeed volatiles from essential oils of *S. molle*, *C. citratus* and *T. filifolia* also reduced attraction to chicha, similar to the reduction in attraction found to oviposition medium (Figure 7B). These tree essential oils were selected for further electrophysiological studies, whereas *S. molle* was used in a field trial (manuscript II).

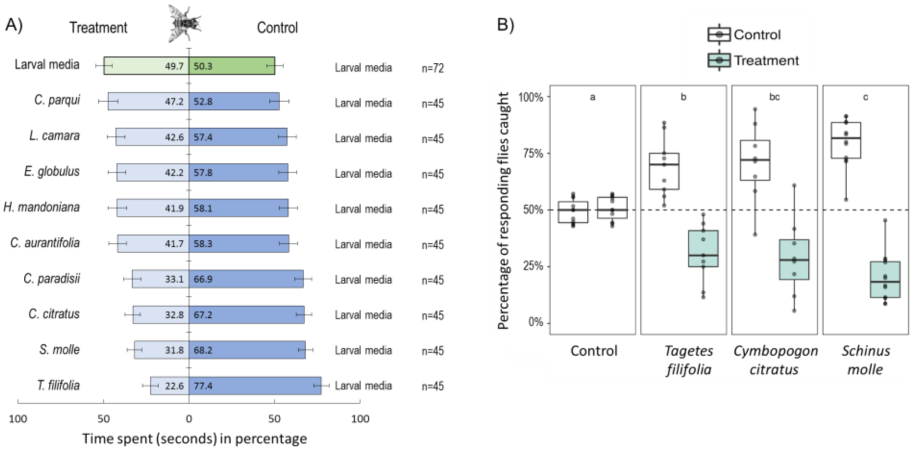

Figure 7. (A) Percentage of time spent in either side of the olfactometer in the treatment and control arm (± SE) for the individually tested flies in T-maze experiments "n" is the number of flies tested. Top green bar depicts control vs control (with a 50:50 distribution) denoting uniformity in the setup, and the subsequent bars represent treatments with different essential oils. (B) Six-choice olfactometer results offering flies a choice between three chambers containing chicha (control) alternated by chambers with chicha + 10 µl essential oil (treatment). Boxplot, with interquartile ranges, median and dots for each observation, shows the percentage of responding flies that were captured in either control or treatment chambers. Different letters indicate differences between treatment ratios (generalized mixed model fitted with a binomial distribution) (figures taken from Manuscript II)

C. citratus and *S. molle* essential oils reduce oviposition of medfly.

We subsequently assessed if *S. molle* also reduces oviposition in fruits in the outside the laboratory. In semi-field settings (Fig. 8A, see description in methodology and manuscript II), fruit positioned next to an eppendorf with essential oil of *C. citratus*, *S. molle* or *T. pusilla* were less preferred than those fruits at a distance. In addition, essential oil suppressed overall suppression, as cages containing an eppendorf with essential oil showed a significantly reduced overall oviposition.

In subsequent experiments in a peach orchard, we found that *S. molle* volatiles released from a waxy matrix, SPLAT (SPLAT-Molle) and applied on leaves in the canopy, reduced fruit damage (2.9%) compared to the control (7.2%), when considering the inner half of each quadrant (p>0.001, GLMM test with binomial distribution, Fig. 8B). Oviposition rates in the outer half of the treated trees did not differ from control trees (p=0.092, GLMM test with binomial distribution, Fig. 8B) (manuscript II).

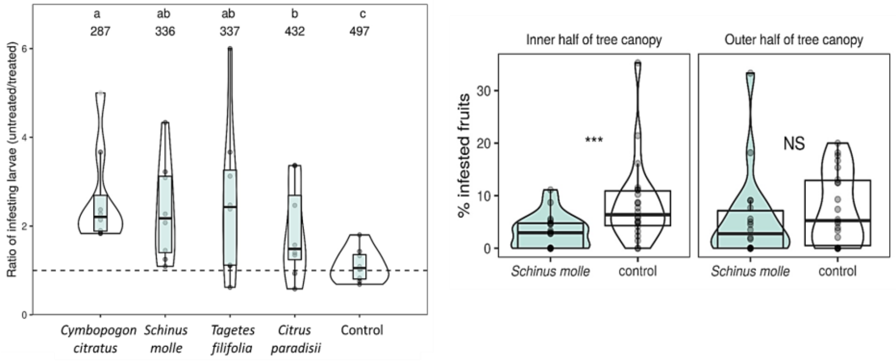

Figure 8. A) Violin plot showing the ratio of larval and pupal infestation in the semi-field experiment in cages. The number above each plot represents the total number of infesting larvae and pupae in each treatment. Treatments with the same letter do not differ significantly from each other (GLMM with a binomial distribution). B) Boxplot and violin plot showing percentage of infested fruits by *C. capitata* of treated trees with SPLAT - *S. molle* oil and untreated trees (control) in the field experiment. Results of inner half and outer half of every tree evaluation is given with the level of significance (GLMM with a binomial distribution).

Repellent essential oils differ in volatile profiles and sensory responses they induce.

A total of 23 unique essential oil volatiles induced responses in *C. capitata*, whereas the maxillary palps detected 14 (Fig. 9). The majority of volatiles were unique for each essential oil, as were the sensory responses they induced, with *C. citratus* most divergent from *S. molle* and *T. filifolia* (Fig. 9). Responses to *C.*

citratus were dominated by the abundant acyclic monoterpenoids, those to *S. molle* by richly represented monocyclic terpenes, and those to *T. filifolia* by anisoles in addition to monocyclic monoterpenoids (manuscript II).

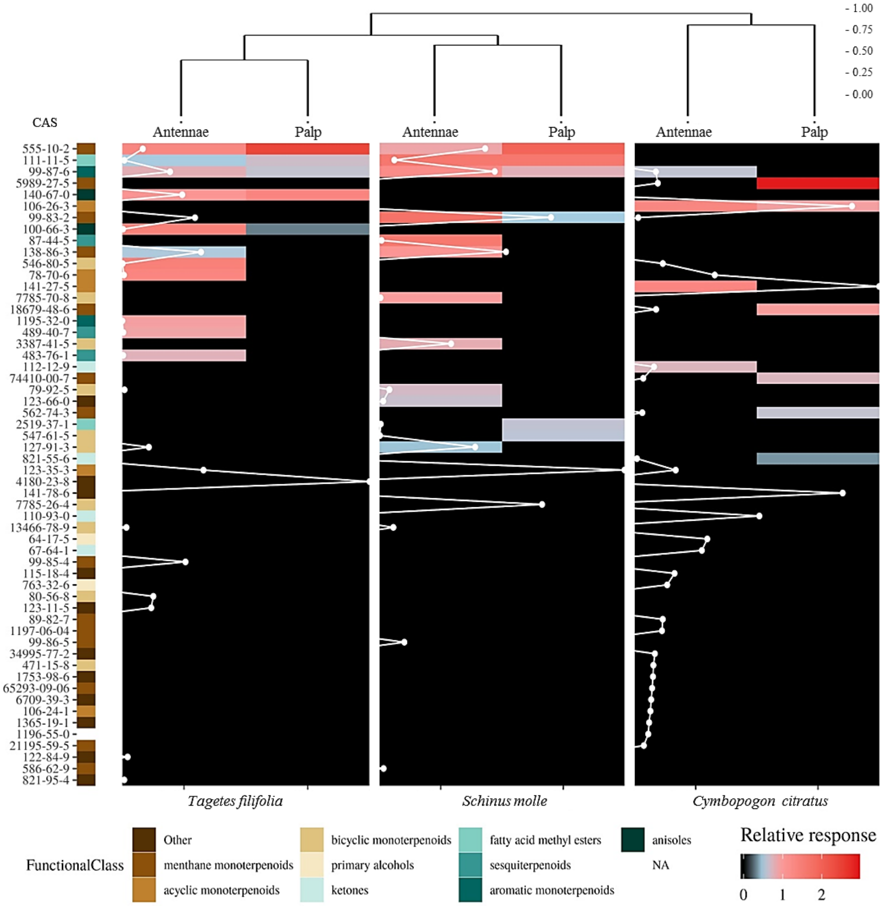

Figure 9. A heatmap of antennal and palpal responses to headspace components of essential oils. From left to right: (i) Tentatively identified compounds represented with their CAS number, (ii) functional chemical classes of the compounds (iii) sensitivity of both sensory organs (antenna and maxillary palp) from female *Ceratitis capitata* to compounds in the tree essential oils (*Tagetes filifolia, Schinus molle* and *Cymbopogon citratus*). The relative response are normalized to the overall response of each recording using a color scalar (i.e., intensity of responses can only be compared within the same column). The white line represents the relative amount of each compound (integration of peaks and normalized to the maximum peak size), absence of a line indicates the compounds not being present in the headspace. The compounds are vertically arranged in decreasing order of distribution in both antennal and palpal response. Code colors of compounds represent the functional chemical class to which every compound belongs. Dendrogram at the top indicate (dis)similarities between electrophysiological responses of both sensory organs to the essential oils from three aromatic plant species.

5.3 Part III: (Dis)similarities in olfactory sensitivities in tephritids to chicha headspace.

A comparative sensory approach to map odor sensitivities across species

Thirty one compounds were tentatively identified from the headspace of chicha, 15 of these were esters (48.4%), three were primary alcohols (9.7%), two benzene and substituted derivatives (6.5%) and the other eleven compounds each represented a separate chemical class. 21 compound were actively detected by antennae and 12 by palpae of the three species of tephritidae (*C. capitata*, *B. dorsalis* and *Z. cucurbitae*), whereas *D. melanogaster* detected 5 in antennae and 4 in palpae (figure 10, manuscript III)

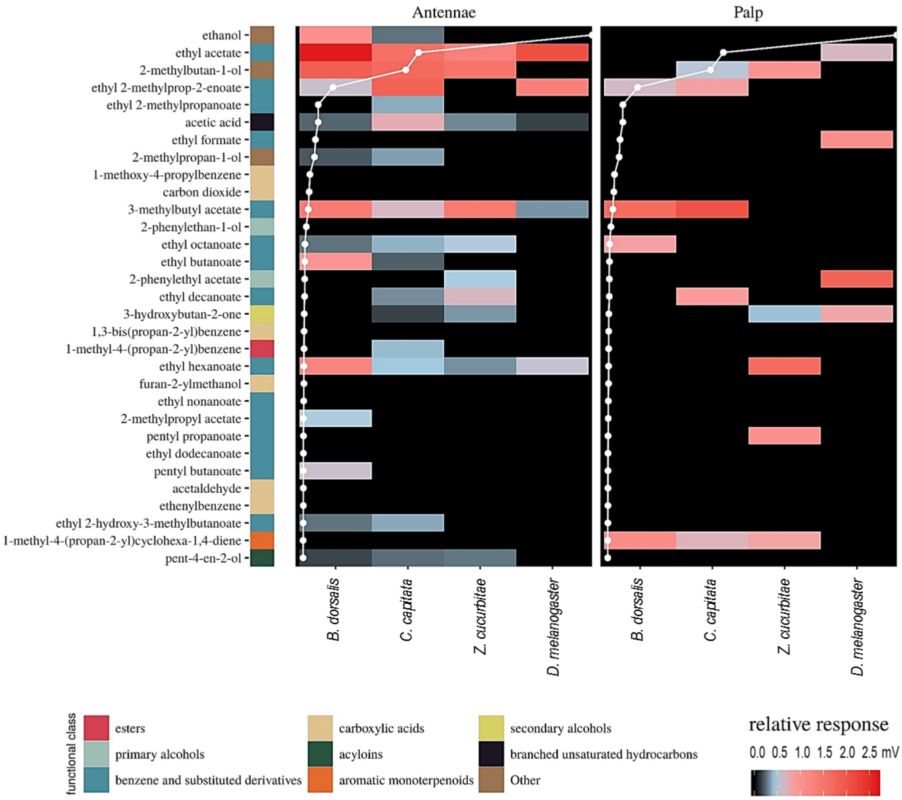

Figure 10. A heatmap over 31 tentatively identified compounds, together they constitute over 90% of the headspace of chicha. From left to right: compound name according to IUPAC and color coded according to chemical class, a heatmap with relative responses, from black (no response) to red in antennae and palp for three species of Tephritidae, *Bactrocera dorsalis, Ceratitis capitata, Zeugodacus cucurbitae*) and unrelated *Drosophila melanogaster*. White line indicate relative amount, according to GC-MS of each of the compounds in chicha.

Fermentation volatiles are significantly correlated with shared fruit volatiles

Next, the species response database to chicha volatiles was appended to an existing fruit-odor olfactome (Biasazin *et al.*, 2018) to screen for evolutionary and ecological signals that connect food and fruit in tephritid fruit flies. A highly significant correlation between chicha and fruit volatiles, and tephritid responses to these were found. Of the 21 compounds that gave an antennal response, 9 were shared with the fruit-odor volatilome. Of these, 7 were detected by the generalist species, *C. capitata* and *B. dorsalis* (Bin(9, 0.1), P<0.0001) whereas 6 were detected by all tephritid species, and 5 were shared among all fruits (Bin(9, 0.017), P<0.0001). Clearly, chicha compounds that induced a response in all fruit flies, are with a highly significant likelihood shared across all fruits (Fig. 11, manuscript III).

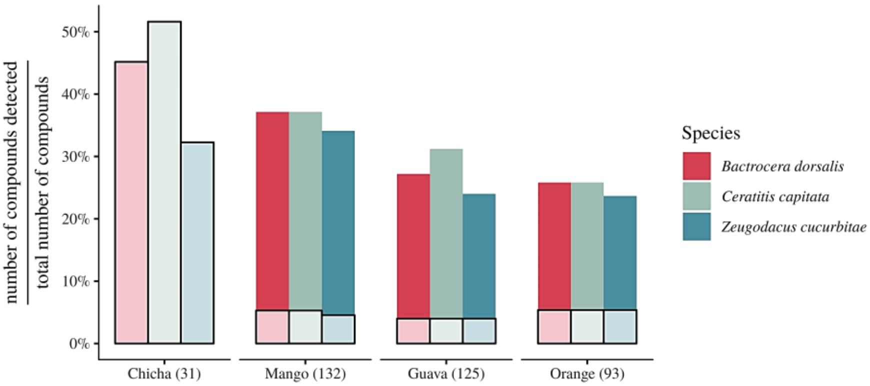

Figure 11. Barplot showing percentage of compounds detected in the antennae of three Tephritidae species (*B. dorsalis*, *C. capitata*, *Z. cucurbitae*) across chicha, and mango, guava, orange and banana. Light-shaded boxes illustrate compounds that are also present in chicha. Number in parenthesis is total number of identified compounds in each sample type.

Chicha volatiles that are antennally active selectively attract medfly in the field

We tested in two consecutive season a blend of 9 antennally-active compounds. In 2017 chicha caught fewer medfly (21.4 FTD- fly per trap per day) compared to 2018 (100.7 FTD). The synthetic blend attracted fewer medflies than chicha (FTD 11 and 18 in 2017 and 2018, Fig. 12A). However, the specificity of the blend was higher (94% being medfly) compared to catches with chicha, which was a broad spectrum attractant (49 and 79% being medfly in 2017 and 2018, Fig. 12B). In addition, catches with the synthetic blend appeared to be more female biased (female/male ratio being 0.69 and 0.59 respectively) compared to chicha (0.52 and 0.48 in 2017 and 2018 respectively, fig. 12C). The synthetic blend, although less attractive overall, was thus more specific and more attractive to females, which are favourable properties (manuscript III).

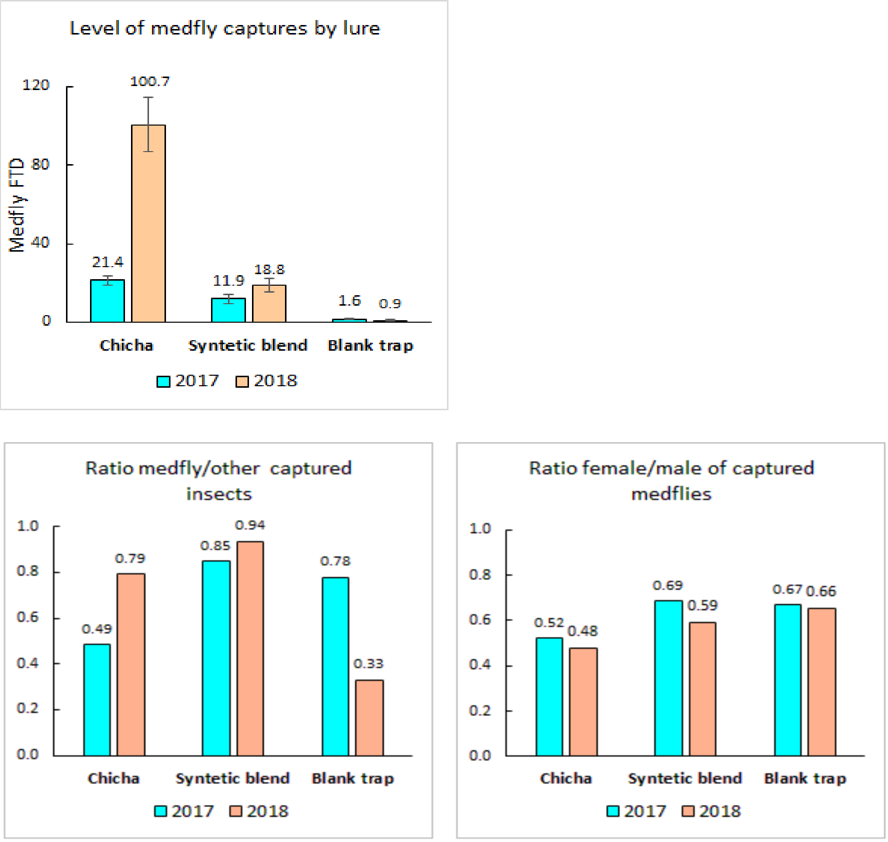

Figure 12. Field evaluation of attraction of a blend of synthetic compounds that induce antennal response in wild *C. capitata*. Comparisons were made with chicha as positive control and a blank trap as negative control in two consecutive fruit seasons (2017 and 2018) A) Level of medfly captures expressed in FTD (fly trap day) B) Ratio of captured medflies over other captured insects, reflect the specificity of the two tested lures. C) Sex ratio of medfly females over males shows the level of attractiveness toward a particular sex group.

6 General discussion

Ethological manipulation as part of IPM programs against fruit flies

In order to reach the one of the 17 sustainable development goals of the United Nations, there is a need to promote the development of novel methods for sustainable agriculture as well as to protect terrestrial ecosystems (Setboonsarng, 2006). In this context, broad-spectrum insecticides need to be phased out and target-specific methods have to be developed that fit with the farmer's IPM toolbox (Deguine *et al*., 2015; Mwatawala *et al*., 2015). Tephritid fruit flies, in particular the invasive generalist *C. capitata* and *B. dorsalis*, have demonstrated a high ecological ability to diverse climatic conditions, and have become a threat in places where fruit production is an important economic activity (Malacrida *et al*., 2007; De Meyer *et al*., 2010). Where established, such as in the Valle Alto, Bolivia (this thesis), farmers are mainly using pesticides. They are though generally aware of the negative side-effects of insecticides, and therefore welcome efforts that contribute to the development of alternative practices to pesticides. In Bolivia some farmers and organizations have attempted to develop IPM strategies, but these appear insufficient to suppress the pest, sometimes exacerbated by the difficulty of application and/or the costs (Mendoza García, 2009; SENASAG, 2010; Pengue, 2016). Novel practical approaches that use local resources, such as the ones reported in this thesis (manuscript I, II), may be integrated into IPM to subsequently reduce fruit flies population levels below the economical threshold.

One of such strategies is the attractant-based approach reported in paper I, a trapping system using chicha as bait in traps designed from plastic bottles. Although this system was developed and tested for the particular context of Valle Alto, Bolivia, its features of easiness and practical handling by farmers, make it interesting for use in other contexts of fruit production threatened by tephritids attack. Chicha is a popular local beverage in the rural area of Bolivia, and is also known to be very attractive to insects, but it has not been used directly as food bait for fruit fly trapping in the field (Mendoza García, 2009). Our results demonstrated that this beverage was more attractive for fruit flies than other commercial food baits or commonly used fermentation and protein based lures tested in this study (Figueroa Candia *et al.*). The attractiveness of chicha was also complemented with the development of a suitable trap from waste materials such as plastic water bottles. The possibility of using chicha and T-traps in monitoring and suppression of *C. capitata* needs to be further tested in field trials and further optimized using participatory research with stakeholders.

In the second manuscript, we tested the potential of aromatic essential oils (EA) in reducing the impact of the medfly. Essential oils have been tested for control strategies for pests and diseases of particularly parasites of humans and animals. However, the potential of employing the repellent feature of these plant secondary substances in suppression of agricultural pest has been little explored (Deletre *et al.*, 2016; Lee, 2018). Our study shows a reduction in attraction of *C. capitata* adults when exposed to the odors of the tested oils in combination with food and oviposition substrates in the laboratory. Subsequent semi-field and field trials showed that the local available essential oil of the Peruvian pepper tree *Schinus molle* (Anacardiaceae) was able to reduce oviposition and the fruit infestation. Although the sensory mechanisms underlying repellence are still not well understood, this is a good beginning for a new line of research in IPM of tephritid fruit pests, essential oils as repellents for agricultural pest. The potential of repellents in organic fruit production and in control schemes for urban backyards deserves attention in future research.

Our results on both repellents (*Schinus molle*) and attractants (chicha) in combination with locally constructed traps, warrants further field studies that test the possibility of developing a push-pull scheme against *C. capitata*, using spatial repellents such as *S. molle* in conjunction with the aforementioned chicha-baited T-trap (Cook *et al.*, 2007; Delrio *et al.*, 2010), to suppress medfly populations and damage below economic thresholds. The techniques are easily employed, traps easily constructed, and material (plastic bottles) and odour sources (chicha) widely available in the region.

Although both the attractant and repellent approaches were developed for the local-context of Valle Alto in Bolivia, the results may instigate researchers to find alternative lures and aromatic plants that can be similarly employed in other geographic locations. Further, the development and refinement of synthetic attractive blends based on chicha, may be more broadly useful in other contexts where fruit flies are of economic importance.

Understanding the evolutionary ecology of tephritids

Tephritid fruit flies are economically among the most important pest species that threaten fruit production worldwide. Currently, most control strategies for this pest are focused on broad-spectrum chemical insecticides frequently in combination with food attractants that are generally attractive to many insect taxa (Revis *et al*., 2004; Stark *et al*., 2004; Peñarrubia-María *et al*., 2014). To date, a lot of research has been done on behavior and ecology of the most important species, such as the generalist *B. dorsalis* and *C. capitata*, often with as goal to find ways to enhance the competitiveness of sterile males in Sterile Insect Technique (SIT) (Shelly, 1999; Hendrichs *et al*., 2002; Benelli *et al*., 2014; Haq *et al*., 2014). However, little research has focused on the evolutionary ecology of the sense of smell of tephritids (Biasazin *et al*., 2018). By studying tephritid olfaction, we could address fundamental questions regarding the evolutionary ecological underpinnings of the preference of tephritids to fruits instead of decaying matter, or the divergence in olfactory sensitivities of generalist and specialists. We identified key compounds that are detected by tephritids in fruits, food attractants, and possible plant-based repellent. The data were further analysed in the context of an expanding olfactome database of tephritid fruit flies that aims at unravelling the evolutionary ecological history of the sense of smell in group of insect (Biasazin *et al*., 2018 and database available in www.tephri.org). This revealed highly interesting patterns. For instance, chichi volatiles, whose detection are shared by three tephritid species (frugivorous in the larval state) and *D. melanogaster* (saprophagous in the larval stage), appeared highly attractive. In addition, these volatiles were highly significantly correlated with compounds that flies detect from ripe fruits, suggesting that these volatiles may have formed volatile bridges from where ancestral saprophily of tephritids evolved a preference for oviposition into fresh fruits (Korneyev, 1999; Díaz-Fleischer *et al*., 2001)

Most studies on insect olfaction focus on the antenna as the main sensory organ related to odor detections (Hansson and Stensmyr, 2011; Dweck *et al*., 2016), however, the physiological and functional role of tephritid maxillary palps in olfactory detection is still a subject of contention (Zhang *et al*., 2011; Chieng *et al*., 2018). Some recent studies revealed that palpae of *Bactrocera* species are strongly related to detection of male lures such as raspberry ketone, cuelure and methyl eugenol (Verschut *et al*., 2018; Park *et al*., 2018; Chieng *et al*., 2018). Here we showed, through gas chromatography coupled palpographic detection (GC-EPD), that palpae of tephritid fruit flies detect volatile compounds from food and essential oils, showing a substantial overlap with those detected by antenna. Whether overlap in palpal and antennal sensory neuron profiles reflects some form of redundancy coding, or whether these sensory neurons are required to induce separate behaviors, is a matter of conjecture (De Bruyne *et al.*, 1999; Stensmyr *et al.*, 2012; Dweck *et al.*, 2016). Regardless, our research does show that palpal sensory neurons converge onto a set of volatiles involved in the detection of food and hosts that strongly overlap with those detected by the antennae.

Understanding the sense of smell in an evolutionary-ecological and behavioral context, supports finding novel techniques that can be developed for new sustainable methods of ethological control in the field (Biasazin *et al.*, 2018). For instance, an attractive synthetic blend based on the detected volatiles by tephritids, may increase the specificity of the lure for the target insect, and also enhancing attractiveness for females, which, because it is the damaging sex, may reduce oviposition and therefore more directly reduce damage (Díaz-Fleischer *et al.*, 2014; Epsky *et al.*, 2014). Regarding repellents on the other hand, a full mapping of the volatiles of these aromatic oils along with the sensory responses to these, may be used to design synthetic repellent mixes that may be more effective than the essential oils on which they were based. The fact that the headspace of the three essential oils differed in the main compounds as well as in the sensory responses they induced, may indicate that different sensory channels underlie these responses (Deletre *et al.*, 2016) and therefore may complement or synergize each other. Future experiments should thus test potential synergy between these compounds, which could then be used to construct blends of natural products or synthetic compounds to increase the potential of repellence in suppressing infestation rates in the field. In addition, to design effective spatial repellent, the mode of action of such repellents needs further research (Deletre *et al*. 2016)

7 Future perspectives

This thesis indicates that ethological control of the Mediterranean fruit fly in Bolivia may be possible with inexpensive local attractants, bottle traps and natural products. Though tested in the local context, they may be extended to other tephritid species and crop systems. Most interestingly, the results provide a perspective for further research evaluate a combination of both the attractant and the repellent approaches in pest control. Since the mechanisms are entirely complementary, a potential synergy in the reduction of infestation rates may be envisioned. However, many technical and social factors have to be taken in account in the development of a push-pull strategy with these techniques, including the selection of essential oils or synthetic compounds thereof, possible toxicity, dose adjustment and environmental collateral effect, especially for beneficial insects. A future combined strategy also will need to evaluate its feasibility in terms of crop economic revenue, its integration with other IPM activities against tephritids and other pests in various agro-ecosystem settings. The potential of more effective attractants and repellent, needs more attention, particularly in organic fruit production and also in control schemes for urban places where pesticides are often inadequate for mass using.

The results presented here also shows the importance of a strong focus of attention on sensory detection techniques and comprehensive and comparative sensory response analyses. This can strongly support the rational development of more efficient and species-specific lures. Further, comparative data gathered should be used to construct databases across insects to better understand the sensory evolutionary ecology of insects. Although this thesis focussed on medfly, we think that many of the findings are readily translatable to other tephritid species. Future test should compare the results with e.g. generalist-invasive species of the *Bactrocera* and *Anastrepha* complexes. The development of lures that selectively attract those Tephritidae species will be of much value in many places in the world where fruit production is threatened by this pest.

Tephritid fruit flies are a subject of study in many places, owing to their economic importance in fresh fruit and vegetable production, international trade, and safety for either producers or consumers. This thesis contributes to the development of novel locally sustainable control strategies, by searching for lures and repellents and providing a framework for a fundamental understanding of this sense in the context of the fly’s biology, ecology and behavior. The sense of smell is increasingly understood, but its role in pest control is still much underutilized. Considering that most of the complex behaviors of insects are governed by olfaction, we strongly believe that harnessing the sense of smell of insects in novel control techniques has every potential to positively impact the sustainability of the horticultural sector worldwide.

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