Encoding of fruit odours by the peripheral olfactory system in *Drosophila suzukii:*

Fruitprints for host selection and prospects for sustainable management

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<< Fais de ta vie un rêve et de ton rêve une réalité >>

- Antoine de Saint-Exupéry

Summary

The fly *Drosophila suzukii* is an invasive pest responsible for major economic losses in fruit industries. Females lay eggs through a punctured hole in the skin of ripening fruits which otherwise would be available for retail. Since 2010 the fly is included in the World Invasive Species Compendium, developed by CAB international (CABI) and on the Pest Alert List of the European Plant Protection Organization (EPPO) as an invasive agricultural pest threatening fruit production. Intensive research has been carried out to improve current management methods and develop Integrated Pest Management (IPM) techniques. One of the challenges is to develop species specific tools which require fundamental research on species-specific host selection processes. *D. suzukii* is also valuable to study host selection and the mechanisms of host detection in a polyphagous pest insect. It has a fascinating ecology and its close relatedness to one of the most intensively studied biological research models *Drosophila melanogaster* facilitates its study. Using a highly sensitive sensory system, females detect and select host plants to lay eggs in the ripening fruits. Semiochemicals released by these fruits may be used to lure them to traps or disrupt host detection.

The aim of the research presented in this thesis was to determine how host fruit odours are encoded by the peripheral olfactory system in *D. suzukii* to enable host fruit detection and discrimination. The results provide a model of olfactory detection of hosts for polyphagous insects. The key result is that the peripheral olfactory system encodes ripe fruit odours via combinations of only a few classes of olfactory receptor neurons (ORNs). The combinatorial activation of the fruit specific ORNs guides host selection behaviour and enables the flies to discriminate among oviposition substrates. This model offers a novel approach for identifying semiochemicals to use in pest management. It enables a rapid screen of chemicals for potential attraction to oviposition sites by determining if the responses they elicit are part of the fruitprint: the pattern of neuron activity induced by host-fruit odours in the peripheral olfactory system.

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List of abbreviations

Abbrevation	***Name***
1H	1-Hexanol
1O	(*RS*)-1-Octen-3-ol
2,3B	2,3-Butanedione
2H	2-Heptanone
3O	3-Octanol
4M	4-Methylphenol
4P	4-Propylphenol
ab	Antenna basiconic sensillum
B	Benzaldehyde
B	Blueberry fruit
BC	Beta-cyclocitral
C	Cyclohexanone
c	Coeloconic sensillum
D. melanogaster	*Drosophila melanogaster*
D. suzukii	*Drosophila suzukii*
DHC	Dynamic headspace collection
DMNT	(*E*)-4,8-Dimethyl-1,3,7-nonatriene
E2H	(*E*)-2-Hexenal
EA	Ethyl acetate
EAG	Electroantennography
Ebe	Ethyl benzoate
Ebu	Ethyl butanoate
EL	Ethyl lactate
F	Fenchone
G	Grape fruit
GC	Gas chromatography
GLV	Green leaf volatile
GR	Gustatory receptor
IA	Isoamyl acetate
IPM	Integrated pest management
IR	Ionotropic receptor
LG	Lagrein grape cultivar
LN	Local interneuron
M	Merlot grape cultivar
MP	Methyl propionate

MS	Mass spectrometry
MSa	Methyl salicylate
NMR	Nuclear magnetic resonance spectroscopy
O	Orange fruit
OBP	Odour binding protein
OR	Olfactory receptor
ORCO	Olfactory co-receptor
ORN	Olfactory receptor neuron
P	Pinot noir grape cultivar
PA	Pentyl acetate
Pb	Palp basiconic sensillum
Pn	Projection neuron
PO	Paraffin oil
R	Raspberry fruit
RL	(*R*)-Linalool
RSL	(*RS*)-Linalool
S	Strawberry fruit
sac	Sacculus
Sc	Schiava grape cultivar
SL	(*S*)-Linalool
SSR	Single sensillum recording
Su	6-Methyl-5-hepten-2-one (sulcatone)
SWD	Spotted wing drosophila
t	Trichoid sensillum
T	Tomato fruit
Tr	Traminer grape cultivar
UK	United Kingdom
USA	United States of America
VOC	Volatile organic compound

CABI	Cab International	https://www.cabi.org/
FEM	Fondatione Edmund Mach- Istituto Agrario di San Michele all'Adige	https://www.fmach.it/eng
EPPO	European Plant Protection Organization	https://www.eppo.int/
EMR	East Malling Research	http://www.emr.ac.uk/

List of chemicals

Compound	***CAS number***
2,3-Butanedione	431-03-8
Benzaldehyde	100-52-7
Geranyl acetate	105-87-3
Methyl salicylate	119-36-8
(*E*)-2-Hexenal	505-57-7
(*E*)-4,8-Dimethyl-1,3,7-nonatriene	19945-61-0
(*E*)-Ocimene	3779-61-1
(*R*)-Limonene	5989-27-5
(*RS*)-Linalool	78-70-6
(*R*)-Linalool	126-91-0
(*Z*)-3-Hexen-1-ol	928-96-1
(*Z*)-3-Hexenal	6789-80-6
1-Heptanol	111-70-6
1-Hexanol	111-27-3
(*RS*)-1-Octen-3-ol	3391-86-4
1-Octanol	111-87-5
2-Butanone	78-93-3
2-Heptanone	110-43-0
2-Isobutylthiazole	18640-74-9
2-Methylbutyl acetate	624-41-9
2-Phenylethanol	60-12-8
3-Hydroxy-2-butanone	513-86-0
3-Octanol	589-98-0
4-Decalactone	706-14-9
4-Ethylacetophenone	937-30-4
4-Methylphenol	106-44-5
4-Pentenyl acetate	1576-85-8
4-Propylphenol	645-56-7
6-Methyl-5-hepten-2-ol	1569-60-4
6-Methyl-5-hepten-2-one	110-93-0
Acetophenone	98-86-2
Benzyl acetate	140-11-4
Butyl acetate	123-86-4
Butyl butanoate	109-21-7
Cyclohexanone	108-94-1
Diethyl succinate	123-25-1
Ethyl acetate	141-78-6
Ethyl benzoate	93-89-0
Ethyl butanoate	105-54-4
Ethyl isobutanoate	97-62-1
Ethyl isopentanoate	108-64-5
Ethyl lactate	97-64-3
Fenchone	1195-79-5
Hexanoic acid	142-62-1

Hexyl acetate	142-92-7
Hexyl butanoate	2639-63-6
Hexyl hexanoate	6378-65-0
Isoamyl acetate	123-92-2
Isoamyl alcohol	123-51-3
Isobutyl acetate	110-19-0
Isopropyl butanoate	638-11-9
Isopropyl propanoate	637-78-5
Methyl (*E*)-2-hexenoate	22210-20-4
Methyl butanoate	623-42-7
Methyl hexanoate	106-70-7
Methyl isopentanoate	556-24-1
Methyl pentanoate	624-24-8
Myrcene	123-35-3
n-Butyric acid	107-92-6
Nerol	106-25-2
Nitropentane	628-05-7
Octanal	124-13-0
Pentyl acetate	628-63-7
Prenyl acetate	1191-16-8
Propyl acetate	109-60-4
sec-Butyl butanoate	819-97-6
α-Pinene	80-56-8
α-Terpineol	98-55-5
β-Cyclocitral	432-25-7
Paraffin oil	8012-95-1
Hexane	110-54-3
Diethyl ether	60-29-7

Synthesized or purified ([1]) from plant extract at Rothamsted Research by David Withall:

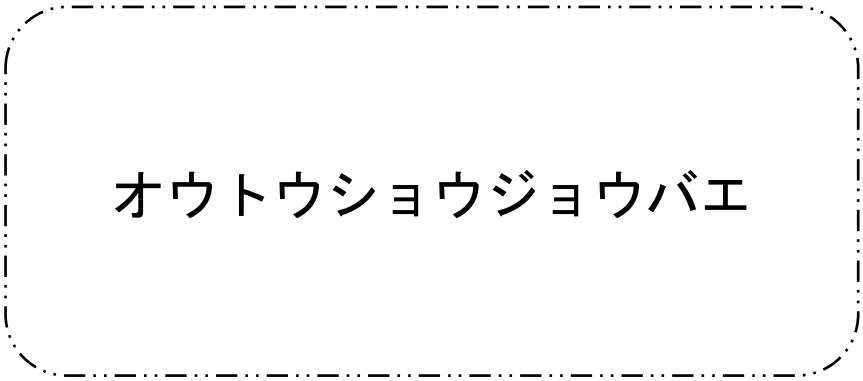
Isopropyl pentanoate

Ethyl-3-methyl-2-butenoate

(1*R*,4a*S*,7*S*,7a*R*)- Nepetalactol

(*S*)-Linalool[1]

1 GENERAL INTRODUCTION



Japanese katakana characters. Pronounced [suzuki shôjôbae]. Translated: yellow peach fruit fly.

1 GENERAL INTRODUCTION

1.1 DROSOPHILA SUZUKII

1.1.1 Identity and description

1.1.1.1 Taxonomy

Drosophila suzukii [Arthropoda, Insecta, Diptera: Drosophilidae, Sophophora] was originally named the cherry fruit fly*, Leucophenga suzukii* by Dr Shounen Matsumura who described it in 1931 from observations in ripening cherries (*Prunus avium*) in 1916 in Yamacho, Yamanashi prefecture, Japan (Matsumura 1931; Kanzawa 1935; Hauser 2011). In most literature it is referred to as *Drosophila suzukii* Matsumura and is commonly called the Spotted Wing *Drosophila* (SWD).

1.1.1.2 Morphology

Taxonomic keys are available for *D. suzukii* (Hauser 2011; EPPO 2013). The following species-specific characteristics were used in this study to identify field collected specimens (Figure 1-1). Adults are small flies approximately 2-3 mm (males) to 3-4 mm (females) long with a wingspan of 6–8 mm. Their head and body are yellow brownish, with large compound red eyes and black stripes on the dorsal side of the abdomen. Adult males possess a black spot on each wing, located on the distal tip near the vein R2+3, and two sets of tarsal sex combs.

Females have a sclerotized ovipositor with strong, contrastingly black marginal teeth. Each ovipositor valve bears 30–36 teeth (Atallah *et al.* 2014). Similar sclerotized ovipositors are exhibited by some other species of the Suzukii subgroup such as *Drosophila subpulchrella*, *Drosophila pulchrella* and *Drosophila immacularis*. However, the ovipositor of *D. suzukii* was the only one reported to be sharp enough to pierce the undamaged skin of ripening soft fruits, notably of blueberry and grapes (Takamori *et al.* 2006; Atallah *et al.* 2014). This evolution of the ovipositor conferred a significant advantage for *D. suzukii* to explore a novel ecological niche consisting of ripening fruits still attached to the plant instead of overripe or damaged fruits that had fallen and represent a niche for many other Drosophilids (Mitsui *et al.* 2010; Atallah *et al.* 2014; Karageorgi *et al.* 2017). Evolution of the ovipositor is a key innovation which also permitted many speciation events in the Tephritoidea. Females could deposit eggs in novel hosts that were inaccessible to others. This innovation contributed to the diversification of species from this family (Díaz-Fleischer *et al.* 2000).

Eggs are milky white and glossy, semi-transparent, on average 0.62 mm long. The eggs have two subapical respiratory tubes, which stick out of the puncture hole in the fruit skin

and are visible under a magnifying glass. The larvae are white to cream in colour with visible internal organs and black cephalo-pharyngeal skeleton, with two protruding black mouth hooks. The pupae are creamy initially and then turn brown in colour and are about 3 mm long by 1 mm wide Kanzawa (1939).

1.1.1.3 Life cycle

A female may lay 1–3 eggs per oviposition site and up to 20 eggs per day. Successful attempts at laying eggs depends on the fruit type, maturity and the number of females competing (Mitsui *et al.* 2007, Kanzawa 1939, Tochen 2014). Through a punctured hole made with its saw-like ovipositor, eggs are laid under the fruit skin allowing the hatching larvae to directly start feeding upon the fruit flesh. There are three larval instars before a pre-pupation stage. Pupae were found on the fruit and in the soil, but no precise quantifications are available. Development time from egg to emergence as adult is approximately ten days at 25 ºC but the pupal period may last 4 to 43 days.

Flies are active from early spring until late summer and autumn. Adults are mostly found to be active around dawn or dusk in cultivated areas (Mitsui *et al.* 2010; Jaffe and Guédot 2019). Up to thirteen generations have been observed within a year with so-called winter-morph adults found during late autumn and winter (Mitsui and Kimura 2010; Walsh *et al.* 2011; Zerulla *et al.* 2015; Rossi-Stacconi *et al.* 2016). These winter-morph adults are characterised by darker coloured bodies and are sexually immature. Females carry immature oocytes and males do not produce sperm (Mitsui and Kimura 2010; Dalton *et al.* 2011; Tochen *et al.* 2014; Grassi *et al.* 2017). This reproductive diapause appears to be induced by environmental changes such as lowering temperature. Overwintering adults can sustain a long pupation stage and live longer in low temperature (i.e. up to 88 days at 10 ºC) and therefore survive until the rise of temperature in early spring (Kanzawa 1939; Dalton *et al.* 2011; Tochen *et al.* 2014).

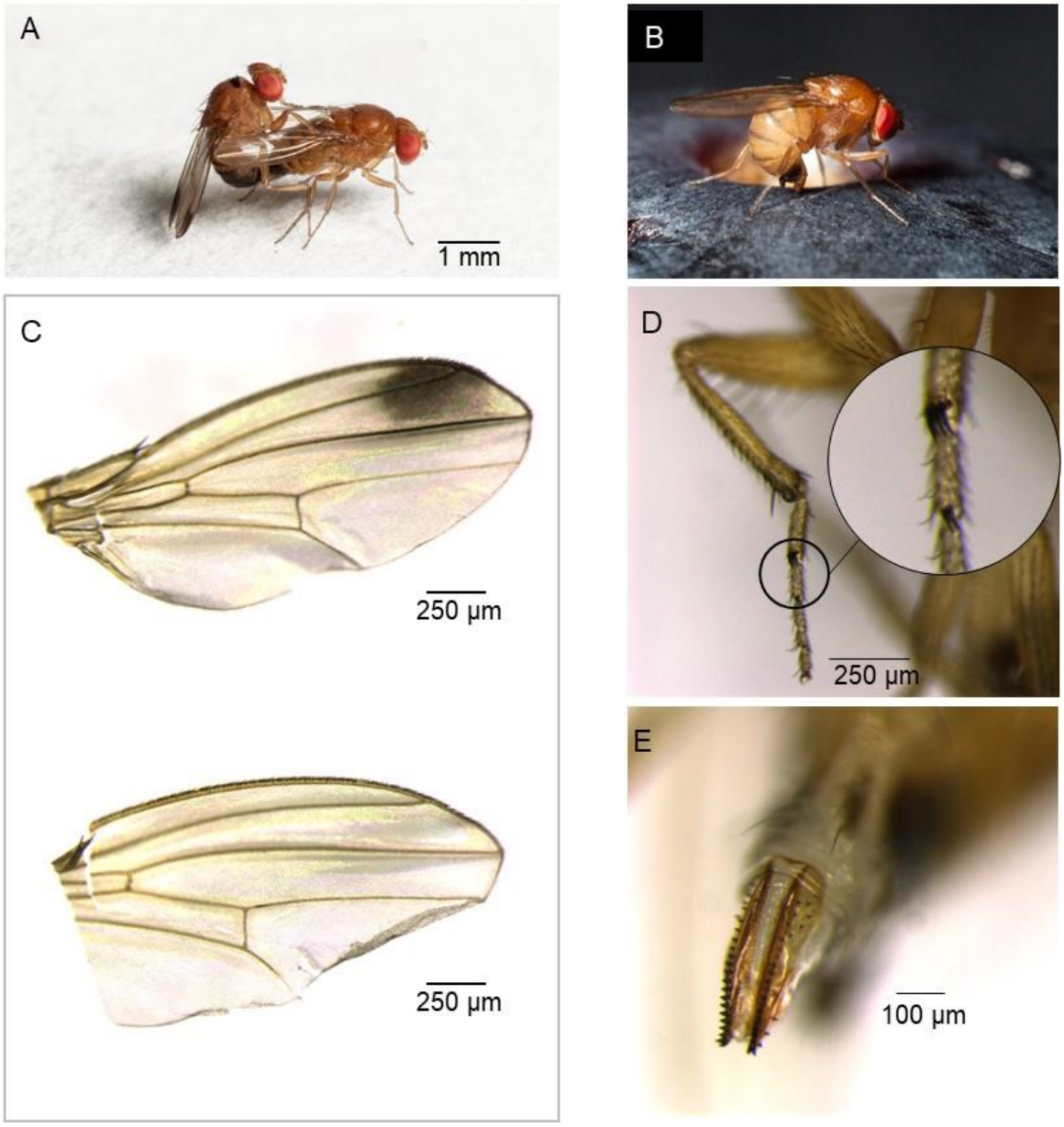


Figure 1-1 Behavioural and morphological features of adult D. suzukii
A) *D. suzukii* mating showing male and female morphologies B) Attempted oviposition on a ripe blueberry with undamaged skin. C) Ventral side of male (top) and female (bottom, some damage) wings. D) Sex combs on the first tarsi of a male. E) Sclerotized ovipositor of a female. (A) and (B) by Graham Shephard (Rothamsted Research).

1.1.2 Ecology for successful invasion

1.1.2.1 Host range

D. suzukii is found on fruits of more than 50 species from at least 18 plant families (Kanzawa 1939; Mitsui *et al.* 2006; Walsh *et al.* 2011; Cini *et al.* 2012; Poyet *et al.* 2015; Kenis *et al.* 2016). They prefer ripening from Overripe fruits unlike most of their closely related *Drosophila species* (Karageorgi *et al.* 2017). Plants were ranked by the amount of damage

from ovipositing females and feeding larvae observed on their ripening fruits thereby classifying them as host, lesser host and non-host species (Mitsui *et al.* 2006; Walsh *et al.* 2011; Bellamy *et al.* 2013; EPPO 2013; Asplen *et al.* 2015; Lee *et al.* 2015; Poyet *et al.* 2015; Kenis *et al.* 2016).

Adult feeding and larval feeding are quite different. Adults were observed drinking the juice from damaged fruits, nectar and tree sap (Kanzawa 1939; Bellamy *et al.* 2013). Larvae feed from the flesh of the ripening fruit upon hatching until pupation. The nutritional needs of the larvae may be acquired from the fruit flesh or from associated microorganisms (e.g. yeast) as is the case in other Drosophilids (Becher *et al.* 2012). Adults were reported to vector microorganisms and predominantly the yeast *Hanseniaspora uvarum* to which they are attracted (Hamby and Becher 2016; Lewis *et al.* 2018). Several yeast species were also identified in the fruits from which larvae were feeding and were shown to be relevant to both feeding and oviposition in adults (Bellutti *et al.* 2018; Spitaler *et al.* 2018).

The fruit flesh would be inaccessible if the female did not puncture the skin to lay eggs underneath. The female host selection thus reflects to some extent the larval host range even though numerous observations showed that the two were not always correlated in phytophagous insects, including in *D. suzukii* (Wiklund 1975; Larsson and Ekbom 1995; Poyet *et al.* 2015; Alhmedi *et al.* 2019). All plant species with damages observed have been detailed in freely accessible reports from the EPPO and CABI websites (CABI 2018; EPPO 2018; Balmès and Mouttet 2019).

1.1.2.1.1 Fruit hosts

Rosaceae, containing an important number of stone and berry fruits, is the plant family that is most affected by *D. suzukii* oviposition with several highly favoured host fruits such as *Prunus spp*. (e.g. cherry, apricot, plum, peach), *Rubus spp*. (e.g. raspberry, blackberry), *Fragaria sp*. (strawberry). The Ericaceae family is also impacted with major damage in blueberry and cranberry.

When local or minor damage has been reported, fruits were considered lesser hosts: less commercially grown Rosaceae (e.g. wild cherry, Asian plum), Actinidiceae (e.g. hardy kiwi) and Moraceae (e.g. figs) are examples (Dreves *et al.* 2009). The amount of damage observed may be due to the commercial nature of the crop with a larger surface coverage compared to wild plants. Other families, including Cornaceae and Garryaceae were only reported in Japan (Mitsui *et al.* 2006). Several wild plants from Northern France have also been added to the list of hosts for *D. suzukii* (Poyet *et al.* 2015).

1.1.2.1.2 Susceptibility of cultivars

Damages have been observed on some cultivars of grape *Vitis vinifera* (Vitaceae) depending on fruit skin thickness and maturity in many wine producing regions of South Europe and North America (Rouzes *et al.* 2012; Baser *et al.* 2015; Ioriatti *et al.* 2015; Pelton *et al.* 2017; Entling *et al.* 2019). Not all cultivars can support the larval development with only less than 10% of the eggs becoming adults (Lee *et al.* 2011). Nonetheless, the oviposition attempts induce significant damages on the fruits and alter wine quality (Ioriatti *et al.* 2015). The host status of grape cultivars is related to the ease by which females can penetrate the fruit skin with their ovipositor. A negative correlation between infestation and penetration force required to pierce the skin was found. Similarly, higher infestation rates in some blueberry cultivars were negatively correlated with the penetration force required to pierce the fruit skin (Kinjo *et al.* 2013; Lee *et al.* 2016). Infestation of fruits also increases with the sugar content and the decreased acidity of the fruits that are associated with ripening of the fruits and with cultivars (Ioriatti *et al.* 2015).

1.1.2.1.3 Hosts thorough the year

D. suzukii can survive and reproduce without ripening fruits around. Overripe fruits, flowers (e.g. Stylax flowers, *Camellia japonica L*. flowers) and damaged fruits (e.g. apples and oranges) serve as suitable food substrate for both adult and larvae (Kanzawa 1935; Kanzawa 1939; Mitsui *et al.* 2006; Mitsui and Kimura 2010). Approximately twenty more substrates among wild plants (fruits, flowers, tree saps) have been identified as suitable for adult and larval feeding hereby showing the large diversity and range of hosts. These are thought to help the flies to go through the year navigating between different food sources as their fruit ripens and become available. (Poyet *et al.* 2015; Kenis *et al.* 2016; Little *et al.* 2017; Elsensohn and Loeb 2018). The more temperate microclimate of woodlands surrounding cultivated areas are thought to be widely used as overwintering sites where numerous alternative hosts can be found (Dalton *et al.* 2011; Grassi *et al.* 2011; Pelton *et al.* 2016; Grassi *et al.* 2017).

1.1.2.2 Geographic range

Its classification as a highly invasive species is not surprising given that *D. suzukii* has spread through most continents in the last 50 years. Below is a summary of its spread in the previous decades. All countries having reported *D. suzukii* are detailed with references in freely accessible reports from the EPPO and CABI websites, and are frequently updated (CABI 2018; EPPO 2018).

D. suzukii was first reported in Japan (1931) followed by reports from Korea (1964), some Hawaiian Islands (1983), multiple provinces in India (1989), China and Thailand (1991).

Since the early 2000s the fly was reported as a damage producing insect in fruit orchards: First in Russia (2003), Costa Rica and Ecuador (2005). In Europe it was first captured in Spain (2008) then France and Italy (2009). Simultaneously, crop damages were reported on the USA-Californian coast (2008) and in Canada (2009). In 2010, the fly was reported on grape and berry fruit crops in ten states of the USA. It was later captured in UK (2012), in Northern Europe (notably in The Netherlands and Sweden) and Eastern Europe (in Hungary and Ukraine). Its spread continued despite the application of control measures and heightened awareness. Flies were trapped in cultivated areas in Central Mexico and in Brazil (2015). As of 2018 the fly was also captured in Turkey, Cyprus, Iran and on the Reunion island (Figure 1-2).

1.1.2.3 Population dynamics

D. suzukii most likely spread because of the global fruit trades. Eggs and larvae in fruits may remain unnoticed if not specifically looked for. In addition, its ability to withstand cold temperature made it possible for the fly to survive refrigerated transportation and storage (Rota-Stabelli *et al.* 2013; Cini *et al.* 2014; Tochen *et al.* 2014). Fruit trade is the most probable explanation for colonisation of isolated islands such as Hawaii, Reunion and the Americas where unassisted migration would require a wind-borne crossing of oceans.

The fast expansion within continents also indicates a high rate of population expansion. With short generation times and availability of large patches of various hosts which all have different maturation times, populations can easily grow throughout the year. Migratory patterns along climatic gradients and seasonal ripening of hosts have been revealed from several studies (Mitsui *et al.* 2010; Ometto *et al.* 2013; Cini *et al.* 2014; Arnó *et al.* 2016; Wang *et al.* 2016; Tait *et al.* 2018; Thistlewood *et al.* 2018). For instance, populations have been observed to migrate between different altitudes to escape hot summer temperatures in Japan, and in Italy (around 600m altitude, personal observation) where mark-release-recapture and microsatellite techniques permitted the identification of long distance movements up to 9000m (Mitsui *et al.* 2010; Ometto *et al.* 2013; Tait *et al.* 2018).

1.1.2.4 Climatic conditions

A high tolerance to climatic conditions significantly increases the chance of successfully establishing where hosts are available. High humidity rates favour their development and longevity (more than 20 days). However, they can survive and lay eggs under 40% relative humidity for a couple of days which considerably increases their chances of survival as population during draught (Hamby *et al.* 2016; Tochen *et al.* 2016; Wong *et al.* 2018). Flies can sustain a broad range of temperature from temperate regions (between 15°C and 25°C), including freezing winter temperatures reaching -14°C (Hokkaido, Japan) and

summer temperatures up to 30°C. The spread of *D. suzukii* to the equatorial region and southern hemisphere (Brazil, Reunion) and equatorial regions (Mexico, Middle East and South Asia) indicates that its establishment is likely to occur in all temperate and tropical regions.

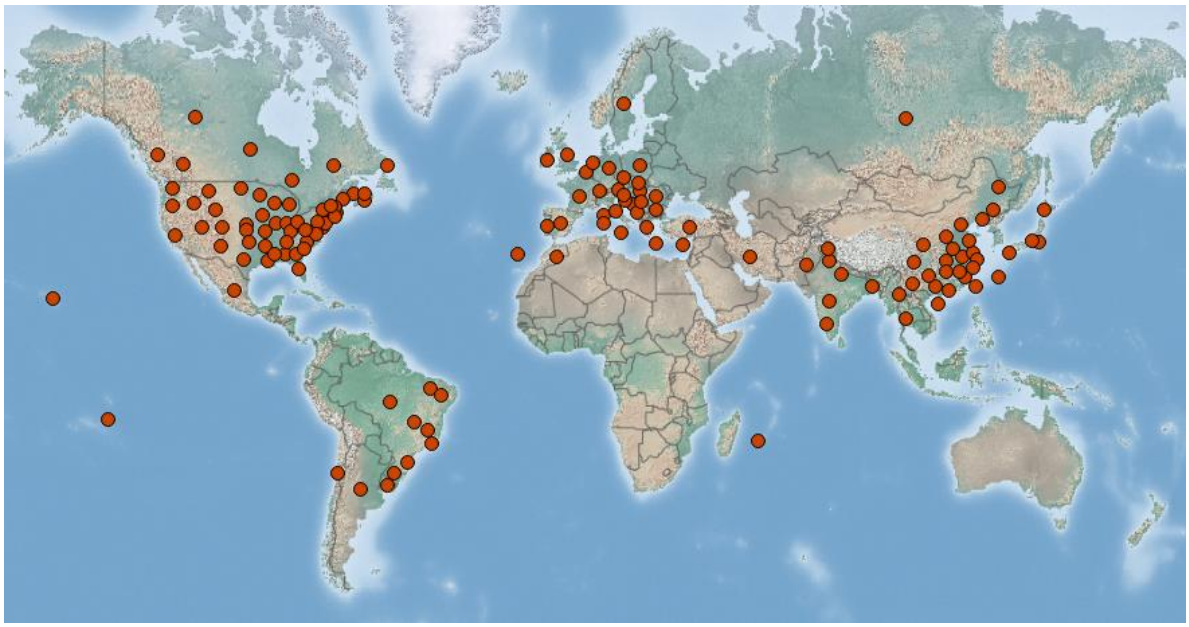


Figure 1-2 World occurrence of D. suzukii as reported in 2019
CABI, 2019. Invasive Species Compendium. Wallingford, UK © Copyright 2019 CAB International.

1.1.3 Management of an invasive agricultural pest

1.1.3.1 Extent of damages

D. suzukii have been included in the invasive Species Compendium developed by CAB international (CABI) as an invasive agricultural pest threatening fruit production worldwide. Following several reports of its presence in Europe since 2008, *D. suzukii* was identified as a threat to fruit production in all European and Mediterranean countries. It was included in the EPPO Pest Alert List in 2010 and is still severely monitored as of 2019 (EPPO 2013; EPPO 2018).

First reports of damage attributed to *D. suzukii* were in cherry with up to 75% of ripening fruits infested on the trees in Japan (Kanzawa 1939). The threat for fruit production was revealed when reports showed over $ 500 million economical losses in cherry production, in USA (Goodhue *et al.* 2011; Farnsworth *et al.* 2016). Examples in Europe include 30-40% crop losses reported in 2010, by local soft fruit growers' associations in the Trentino province in Italy, one of the most productive regions of soft fruits in Europe. Despite pest management, about 7% of revenue losses (industrial output), accounting for losses of

harvest and costs related to the integrated pest management measures in berry production (mainly strawberry, raspberry, blueberry, blackberry) were solely due to *D. suzukii* infestation in 2014 in the same region (Grassi *et al.* 2011; De Ros *et al.* 2013; Del Fava *et al.* 2017).

Many growers currently rely on the use of several classes of pesticide (including pyrethroids and organophosphates) in Europe, Asia and America, which poses several health and environmental concerns, notably their sublethal effects on pollinators and other beneficial insects For instance, pesticide residue levels often reach the health and safety limits imposed on harvested fruits inducing either the risk to sell hazardous fruits or a loss of production when these are refused by retailers. In addition, peaks of infestation often coincide with high humidity rates during which the use of pesticides is obsolete as it is washed away by rain in the soil. This leads to increased usage of pesticides and increased environmental pollution with little efficacy against the pest (Haviland and Beers 2012; Rota-Stabelli *et al.* 2013; Wiman *et al.* 2016). Another concern is that risks of evolving resistance to insecticides are relatively high for a species with a short generation time and with repeated exposure to a limited panel of different insecticide classes (Van Timmeren and Isaacs 2013; Knight *et al.* 2016; Smirle *et al.* 2017).

1.1.3.2 Monitoring and management

An integrated and multidiciplinary pest management approach is necessary to control the fly in its diverse environments as reviewed by Cini *et al.* (2012). The development of knowledge on its biology and ecology are important aspects which would permit to improve sustainable and less pesticide dependent integrated pest management (IPM) strategies for *D. suzukii*. The latter include mating disruption, push-pull, trap and kill (see below) and have proven their efficacy against many other agricultural pests and disease vectors (Cini *et al.* 2012). Notably, an improvement of traps and baits is crucial for the monitoring and reduction of *D. suzukii* in fruit orchards. Current methods when applied early in the growing season can avoid large economic damages but are not enough during peak infestations without pesticide applications. For instance, nearly 40,000 adults have been captured in a single trap from a raspberry grower in Italy (Pers. comm. A. Grassi, FEM). Weekly monitoring in crops and in surrounding woodland patches all year-round permit to predict the rate of seasonal infestation and the efficacy of IPM.

Reviews on the status of *D. suzukii* control and the prospects for different management techniques are available (Walsh *et al.* 2011; Cini *et al.* 2012; Andreazza *et al.* 2017; Anfora *et al.* 2018). Among them, the use of plant and insect originating volatile chemicals for monitoring and control (e.g. attract and kill, push-pull, mating disruption) has showed their

efficacy in many crop pests and is a promising technique against *D. suzukii* (Agelopoulos *et al.* 1999; El-Sayed *et al.* 2006; Reddy and Guerrero 2010). Insights on current management was kindly given by A. Grassi, E. Mescalchin (Fondatione Edmond Mach) and F. Sinn (Südtiroler Beratungsring) in Trentino and Alto Adige provinces, Italy (Figure 1-3).

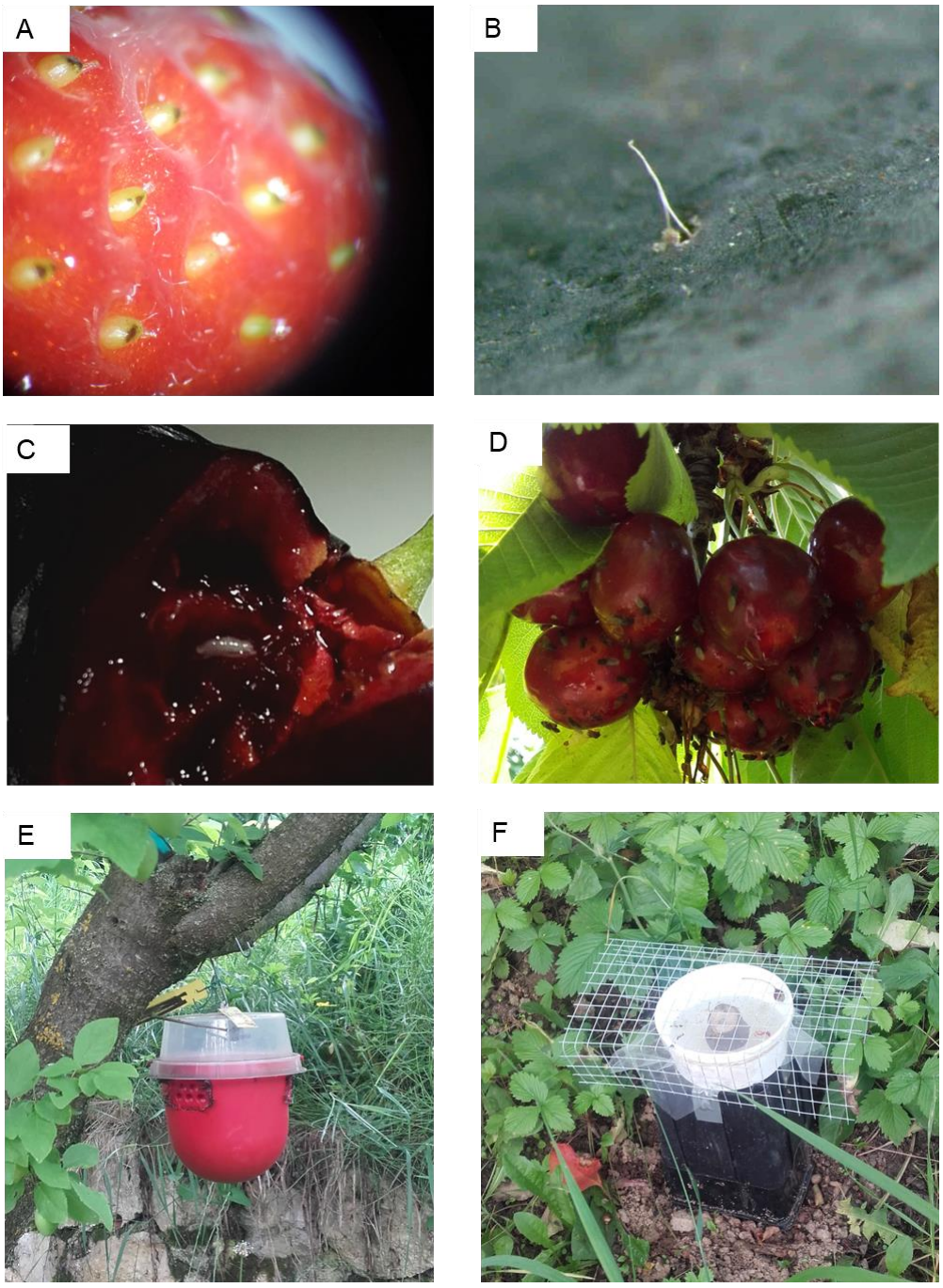


Figure 1-3 Damage, monitoring and control of D. suzukii in Trentino, Italy
Eggs are visible through the skin of ripe strawberry (A) and fig (B). C) late instar larva feeding from the flesh of a cherry. D) Cluster of cherries still attach to the tree infested with various insect species following the damages to the ripening fruits done by *D. suzukii*. E) Red trap with Droskidrink®. F) Baits with *Drosophila* pupae to attract the local

predator *Trichopia drosophilidae* (a pupal parasitoid wasp) monitored to develop as a biological control. Traps were developed by A. Grassi and colleagues (FEM, Italy).

1.2 INTRODUCTION TO HOST SELECTION IN PHYTOPHAGOUS INSECTS

Host selection is the process by which insects identify and reach plants where they can find food, oviposition sites and mates. It can be described as two steps: host finding and host acceptance, both are guided by chemosensory (olfactory and gustatory) and visual cues.

1.2.1 Processes of host selection

1.2.1.1 The role of olfaction

In many species, the first step in host selection rely on olfactory cues, that enable the insect to detect and orient towards potential hosts. Insects recognise host specific cues released among thousands other odours from the habitat via a highly sensitive and specialised olfactory system (Visser 1986; Visser 1988; Hansson *et al.* 1999; Schoonhoven *et al.* 2005; Bernays and Chapman 2007). Olfactory host cues are subject to changing dynamics because their release depends on the plant state (i.e. growing stage, damages, reproductive state) (Bruce *et al.* 2005) and they are carried by multidirectional winds of various intensities (Cardé and Willis 2008).

Habitats in which to find hosts are first detected via the recognition of ubiquitous and/or habitat specific combinations of plant volatiles (Hansson and Christensen 1999; Bruce *et al.* 2005; Schoonhoven *et al.* 2005; De Bruyne and Baker 2008; Bruce and Pickett 2011). As the insect approaches more host-specific cues are being detected (Figure 1-4). At the same time insects are seeking host plants (which can support the larval development into adult) they detect, and process information related to their safety (predators, competitors) and mating possibilities (mate finding via species-specific body odours and pheromone). For instance, insects may associate both food and mating by the detection and processing of a combination of cues (Lebreton *et al.* 2012). The combination of plant kairomone (e.g. a volatile that benefits an individual of another species which receives it and harms the emitter) with female pheromone significantly increase the attractiveness of the Lepidopteran *Cydia pomonella* (Reddy and Guerrero 2004). Another example is the detection of predators inducing the insect to move away from a potential host plant (Ebrahim *et al.* 2015).

1.2.1.2 The role of visual cues

In vicinity of these potential hosts visual cues become an important part of host plant selection, depending on insect species. Colours, patterns and contrasts between host and background play a role in host recognition (Prokopy and Owens 1983; Bernays and

Chapman 2007). *D. suzukii* can be attracted to odourless colours in laboratory bioassays, mainly red, purple and black under the condition of background contrasting (Kirkpatrick *et al.* 2018). Furthermore, olfactory cues and visual cues in association increase the attractiveness of hosts. In *D. suzukii*, host selection appear to necessitate the capacity to process several cues at the same time (Lasa *et al.* 2017; Cloonan *et al.* 2018; Kirkpatrick *et al.* 2018). The visual cortex of *D. suzukii* is one of the largest among Drosophilids and appear to have been positively selected (Keesey *et al.* 2019).

1.2.1.3 Acceptance of hosts

The second step of host selection is the acceptance, which is generally mediated through the gustatory system. Upon landing on the substrate, the insect evaluate food nutritional quality through taste (and perhaps olfactory) receptors on the tarsa, mouthparts and ovipositor (Schoonhoven and Van Loon 2002; Hallem *et al.* 2006a; Vosshall and Stocker 2007). For instance, the detection of host specific chemicals induce subsequent oviposition or feeding in the moth *Manduca sexta* (del Campo and Renwick 2000).

In *D. suzukii*, host acceptance appear dependent on the high sugar content and low acidity for oviposition (Walsh *et al.* 2011). Furthermore, the food-associated microbiome appears to affect the behaviour of adults that are detecting them (Qiao *et al.* 2019). Oviposition success however, depends largely on the accessibility of the fruit: the principal parameter is the thickness of the fruit skin which may be impenetrable (Atallah *et al.* 2014; Ioriatti *et al.* 2015; Lee *et al.* 2016).

1.2.2 Plant semiochemicals

Chemical communication is widely used in species interactions within and between all trophic levels. When released chemicals from any organism affect other organisms (intra and interspecific) in their physiology or behaviour as means of communication, chemicals are referred to as semiochemicals.

1.2.2.1 Plant volatiles

Plant volatile organic compounds (VOCs) are produced in a large variety that depends on the plant species and state (i.e. age, fruit ripeness, stress). VOCs are released by almost all plant tissues and include green leaf volatiles (GLVs), nitrogen-containing compounds and aromatic compounds in addition to many other chemicals. Many are by-products of plant physiology and produced as a reaction to diverse stresses. Many VOCs are involved in intra- and inter-specific communication. Different plant lineages often rely on different sets of VOCs to face similar situations. For instance, the primary defence mechanisms involve the release of toxic and repellent compounds against phytophagous attack. The

secondary defence mechanisms include the release of VOCs that are attractive to the natural enemies of the attacking insect or that are signalling neighbouring plants of a phytophagous attack as reviewed by Schoonhoven (2005). Pollinators and seed dispersing animals detect attractive VOCs which benefits the plant. Plants are also associated with other living organisms such as predators, conspecific and microorganisms. They release body odours, pheromones and other compounds from their activity that can be detected. Phytophagous insects such as *D. suzukii* use semiochemicals to find food and oviposition substrates. Most living entities in the habitat release chemicals from which the insect must identify the relevant cues (Figure 1-4). Further details can be found in many published reviews on plant-plant communication and insect-plant interactions (Lerdau *et al.* 1997; Baldwin *et al.* 2002; Peñuelas and Llusià 2004; Kost and Heil 2006; Baldwin 2010; Holopainen and Gershenzon 2010; Heil 2014; Pickett and Khan 2016).

1.2.2.2 Host cues are mixtures of chemicals

The host insect chemical interaction is a complex yet fascinating system to decipher. The insect needs to have a remarkably sensitive olfactory system to detect very low fluxes (estimated at 10 molecules per second) of chemicals (Baker *et al.* 1998). It also must be tuned to a broad range of chemical classes (e.g. ubiquitous esters and alcohols, taxonomically specific isothiocyanates) and should be able to discriminate and process the informative cues from the habitat (Hansson *et al.* 1999; Desogus *et al.* 2003; Campbell and Borden 2009; Bruce and Pickett 2011).

Taxonomically distant host plants release very distinctive headspace volatiles from which many compounds are detected by insects. Some of these compounds are ubiquitous plant volatiles and some are species specific (Campbell *et al.* 1993; Birkett *et al.* 2003; Bruce *et al.* 2005; Cunningham and West 2008). In natural conditions behavioural responses from phytophagous insects are often induced by mixtures of headspace plant volatiles rather than by single compounds. The composition of these mixtures can provide important cues that allow animals to discriminate potential hosts from non-hosts (Pickett *et al.* 1997; Agelopoulos *et al.* 1999; Bruce *et al.* 2005).

Behavioural responses to mixtures are different from the responses to single compounds and do not always reflect their composition (Bruce and Pickett 2011). Chemicals that are not attractive when presented alone may form an attractive mixture. For instance, a six-components blend attracted the orange wheat blossom midge *Sitodiplosis mosellana* whereas none of the components induced attraction when tested alone in olfactometer assays (Birkett *et al.* 2004). Similarly, a mixture of attractive compounds can be unattractive if the compounds are antagonists. Volatiles may also act as inhibitors or masking cues

rendering a mixture unattractive. For instance, the interaction between a pheromone component (*(Z,E)*-9,11-tetradecadienyl acetate) and the plant released terpene linalool reduced the firing rate of ORNs hereby decreasing the receptivity to sexual signals in *Spodoptera littoralis* (Hanot *et al.* 2009). These examples show the importance of considering host cues as whole mixture of several components including from the background habitat when studying host-insect interactions (Carde and Minks 1995; Schoonhoven and Van Loon 2002; Bruce and Pickett 2011).

1.2.3 Discrimination of host odours in the habitat

Habitats contain thousands of chemicals through which the insect must navigate and find hosts (Figure 1-4). Insects detect some these plant-released chemicals in order to identify which is a host substrate. Many of these chemicals are ubiquitous and a number of which are taxonomically specific (del Campo and Renwick 2000; Dicke 2000; Bruce and Pickett 2011). Finding the rare relevant odour cues in the habitat is a complex task as ubiquitous habitat/host volatiles can be shared by many plants (Campbell *et al.* 1993; Birkett *et al.* 2004; Bruce *et al.* 2005). The habitat dynamics with conspecifics, other species, competitors and predators is also a source of variability which influence the odorant signals originating from host plants (Murlis *et al.* 1992; Gaudry *et al.* 2012).

Furthermore, the odorant plume reaching the insect olfactory system does not necessarily reflect the composition of a mixture that is its source (Figure 1-4). The odour plume represents a plant specific and dynamic signal which can be recognise through the background of habitat chemicals: odours are carried by the wind in unpredictable pulses of different concentrations (Murlis *et al.* 1992; Geier *et al.* 1999). Insects supposedly use timing of pulses encountered and wind direction in addition to the chemical content to orient toward the source of the plume since a concentration gradient seems unlikely over long distances (Cardé and Willis 2008; Renou *et al.* 2019). Furthermore, odour plumes content depends on the plant physiological state (i.e. ripening of fruits, damages by diseases or herbivores), the distance from the insect, and is modulated by the richness of the environment and the climate conditions (Hilker and Schröder 2008).

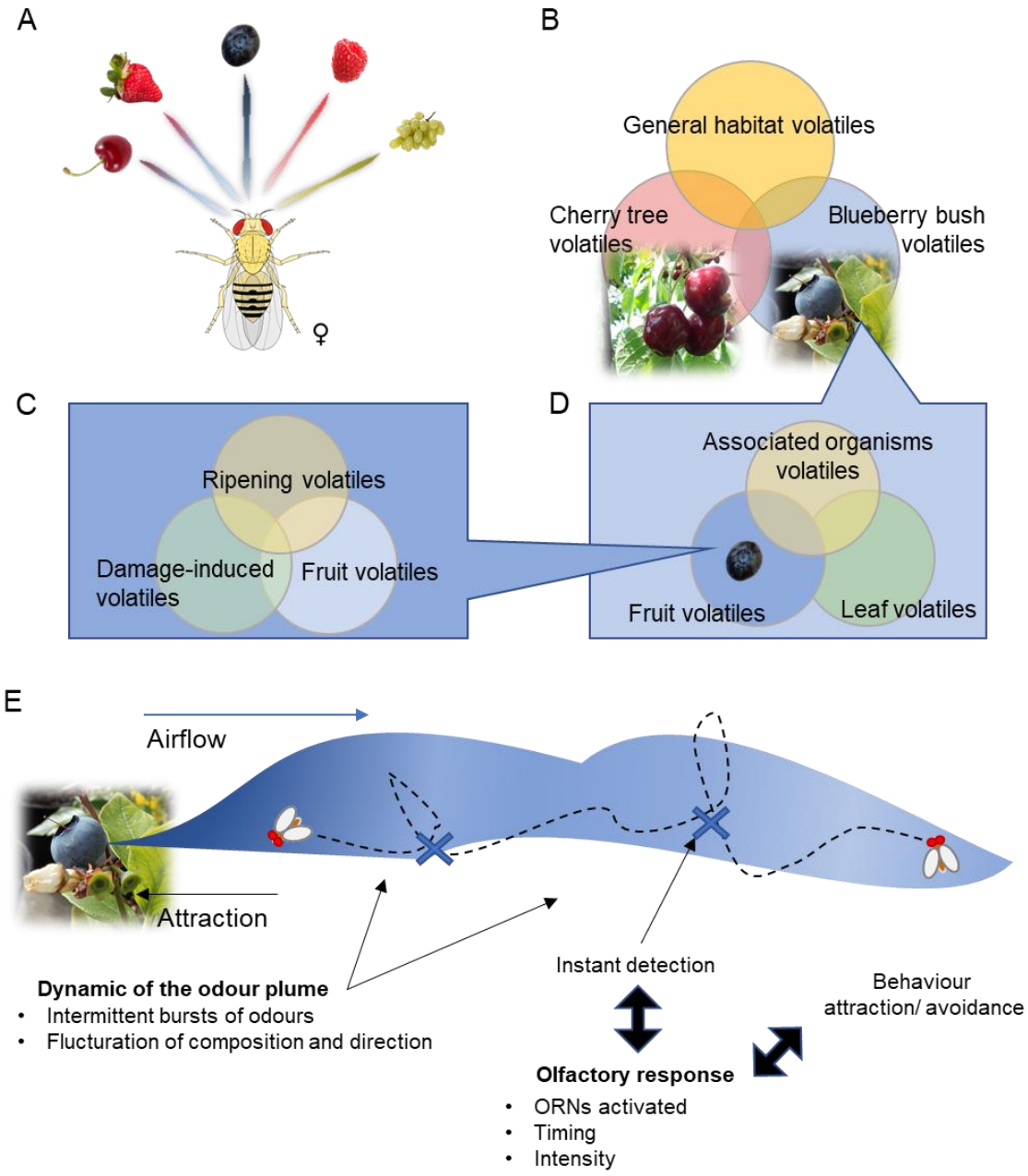


Figure 1-4 Detection of host plant volatiles in the habitat by D. suzukii

A) Illustration of five host fruit volatile plumes detected by gravid female *D. suzukii* in one habitat. B-D) The habitat contains semiochemicals from a multitude of associated biomes. Host selection requires the capacity to recognize host suitability over long distances in this habitat. With multiple suitable hosts female must be able to discriminate and choose a favoured host. B). Detection of the vegetation patch where the host might be localised (e.g. cherry or blueberry). C) Distinction of the suitable host with specific (e.g. blueberry plant) and ubiquitous volatiles. D) Recognition of the oviposition substrate (e.g. fruit ripening stage). E) Dynamic of an odour plume with widening odour trail downwind is an illustration. Intermittent pulses of chemicals (represented by darker shades) may be stochastically distributed within the plume. The plume dynamic and spread may fluctuate with the wind. The behavioural trail is an example trace from *D.*

melanogaster response to attractive odours from fermented banana in a field simulator (Budick and Dickinson 2006). Various odour-guided behaviours exist within and across species, the ones employed by *D. suzukii* are unknown. Hypotheses are from work by van Loon and Schoonhoven, 2005; Bruce and Pickett, 2011; Keesey *et al.,* 2015; Webster and Cardé, 2016, Murlis and Jones, 1981 and reviewed by Renou *et al.* (2019).

1.2.4 Host selection strategies

Hosts in this study are defined as plants whose ripening fruits are susceptible to oviposition (thin skinned) and can support the development of larvae into adulthood. Hosts are differentially attractive as reported by Abraham *et al.* (2015). *D. suzukii* is referred to as a polyphagous fly because of its ability to utilise fruit from taxonomically diverse plants, by opposition with a monophagous insect, feeding on a single plant genus (Schoonhoven and Van Loon 2002). The term generalist is not preferred because it appears that larvae and adults have different feeding preferences (Becher *et al.* 2012; Bellutti *et al.* 2018; Lasa, Navarro-de-la-Fuente, *et al.* 2019). Indeed, the source of the nutrition of the larvae may be the flesh of the fruit or associated microorganisms, making them either generalist on fruits or specialists on yeast which acted as a phagostimulant. Adults are associated with yeasts as other Drosophilids, suggesting they may be specialist feeders on yeast.

1.2.4.1 Host specialisation

In phytophagous insect gradients of specialisation can be found. Some insects are strict specialist i.e. feeding from a small number of taxonomically close plant species, other insects are extreme generalists i.e. feeding from a large selection of taxonomically diverse and taxonomically distant plant species (Bernays 1998). Insects can be ranked by the number of host plant species they can live on from the most specialist to the least specialist (or the most generalist). In *D. suzukii* the range of hosts and their common characteristics (in addition to thin skin) are not fully known (see above). These hosts may share a common characteristic for which the insect would then be a specialist on. For instance, *D. suzukii* may feed on the yeast species *H. uvarum* hence be attracted to its presence on many fruit types. Indeed, the fly growth and fecundity were enhanced when food was supplemented with this yeast species (Bellutti *et al.* 2018; Spitaler *et al.* 2018). It can be suggested that *D. suzukii* may be a specialist feeder on yeast which can be found on fruits from all plant hosts.

Another hypothesis is that generalism can be the outcome of a great intraspecific diversity. Species are composed of several populations that are specialised on a narrower selection of hosts from the range they can sustain (Bolnick *et al.* 2002). In *D. suzukii* several populations may have specialised on a subset of hosts (i.e. raspberry, cherry or blueberry) which they would prefer when given a choice. Alternatively, they may not have preference

for said host type as they are not all simultaneously available. With its various fruit hosts that are consecutively available and ranked accordingly (Abraham *et al.* 2015) *D. suzukii* is an interesting model to tackle the question whether exposure to a host may modulate host preference/rank in a polyphagous fly and perhaps drive intraspecific host specialisation.

1.2.4.2 Advantages of polyphagy

To ensure an optimal foraging technique such as avoiding flying long distances and exposure to predators if oviposition in the substrate is not guaranteed (Mayhew 1997; Cunningham *et al.* 2001), the neural system must be able to perceive and manage various inputs at the same time to rapidly and efficiently recognise and discriminate among diverse potential hosts for a specific goal (e.g. oviposition). Focusing on few stimuli enhance the accuracy of quality assessment hereby conferring an advantage at being a specialist (Dukas 1998). It was for instance illustrated in the aphid *Uroleucon ambrosiae.* Individuals from a population specialist on the giant Ragweed *Ambrosia trifida* (Asteraceae), were faster and better at selecting their host compared to the individuals from a more generalist population found on several other species of Asteraceae (Bernays and Funk 1999). This study illustrated that the processing of cues from several potential hosts may be slower to process. Host selection in polyphagous insects may thus be slower and more prone to mistakes compared to that of specialists, because of a limited neural ability to process large amounts of information in short periods of time (Dukas 1998; Bernays 1999). Hence, some discriminatory cues may be overlooked and with an increase number of stimuli, information may not be optimally processed.

An example of these limitations is that choosing the wrong plant could be fatal for the survival of the individual genetic pool (i.e. wrong oviposition choice). The hypothesis of the preference-performance correlation is that females select an oviposition substrate that would maximize the likelihood of survival of juveniles until adulthood (Jaenike 1982; Mayhew 1997). This correlation is widely discussed and was disproved in several species (Wiklund 1975; Janz 2003) including in *D. suzukii.* The inability of females to discriminate fruits which support the progeny's survival until adulthood was demonstrated on fruits from Prunus spp. For instance, females lay as many eggs on *Prunus padus* (Bird Cherry), *Prunus lusitanica (Portugal Laurel)* and *Phytolacca Americana* (Pokeweed) as they lay on *Rubus fruticosus agg*. Fruits (blackberries and raspberries). While most of the eggs laid on the latter successfully developed into adults, no flies emerged from the other fruit types, in controlled conditions (Poyet *et al.* 2015; Alhmedi *et al.* 2019). Such accidents may increase with the number of attractive substrates which may mistakenly be considered as hosts, hence be more frequent in the most generalist species. Insects also rely on visual cues and

abiotic environmental conditions to select hosts. As explained previously, insects are guided towards host plants using a multitude of cues including olfactory, visual and dynamics (e.g. wind direction, timing of odour detection).

1.2.4.3 Foraging strategies

Insects also adopt foraging strategies to optimise the recognition of hosts. The following may be adopted by *D. suzukii* to discriminate among its multiple hosts.

The first strategy which may take place is to rely on past experiences or known hosts the insect originated from. Females may be keen to favour the host they originated from (as larvae or as previous oviposition substrate) because it is known as suitable hence less risky than novel hosts (Cunningham *et al.* 2001; Cunningham and West 2008). Previously encountered host substrates may thus influence the adult host choice for oviposition (Jaenike 1982). However, the effect of exposure and learning from experience (as in the Hopkin's selection principle) is controversial with refuting (Jaenike 1982; Janz *et al.* 2009) and validating (Davis 2008; Cahenzli and Erhardt 2013; Anderson and Anton 2014) observations, mainly in Lepidoptera.

Another strategy would be for females to be attracted to hosts following a ranking of suitability. A ranking of host substrate is observed in *D. suzukii* (Bellamy *et al.* 2013; Abraham *et al.* 2015). This strategy implies that females would lay eggs in non-suitable fruits only when there are no other options available. These lesser ranked hosts may become new niches if the progeny survives and subsequent generations adapt (Larsson and Ekbom 1995). In addition, a phenomena called "attentional shift" described in blue jay birds and in Swallowtail butterflies (Papaj 1991; Bernays 1996; Dukas and Kamil 2000) states that the individual focuses on one host substrate at a time hereby discarding any others. i.e. females that are laying eggs on a host species, may continually do so until this host is no longer available then another host may be chosen. This scenario of individualised or temporal specialism is possible in *D. suzukii*, given that its ranked hosts have different ripening seasons (Cini *et al.* 2012). Indeed, the females are not often exposed to multiple hosts at once: For instance, the most preferred cherry is ripening before strawberry and blueberry fruits (Abraham *et al.* 2015; Poyet *et al.* 2015; Tait *et al.* 2018).

1.3 *Introduction to the olfactory system dedicated to host/food detection in Drosophila*

In insects, a great diversity of shapes and complexity of olfactory organs allows adaptation to specific hosts. The key role of the olfactory system for host selection was demonstrated via organ and genetic ablation in many insect taxa (Schoonhoven and Van Loon 2002; Galizia and Rössler 2010; Hansson and Stensmyr 2011). Although shaped differently the structure and organisation can be recognised across taxa: the peripheral organs consist of olfactory receptor neurons (ORNs) housed in structural units (sensilla). The binding of odorant molecules to olfactory receptors (ORs), possibly mediated by odorant binding proteins (OBPs), induces a flux of cations across the dendritic membrane. Trains of action potentials are generated and are relayed towards centralised olfactory units (Clyne *et al.* 1997; Shanbhag *et al.* 1999; Dobritsa *et al.* 2003).

A deeper understanding of how semiochemicals are encoded by the olfactory system would be favourable to improve management of insect pests (van der Goes van Naters and Carlson 2006). Olfactory systems in Lepidoptera and Diptera are the most studied not only because of the facility with which they allow to explore fundamental scientific questions but also because of their economic importance: many are agricultural pests and Diptera also include several mosquitoes and flies that are vectors of life-threatening parasites and viruses. The fly *D. suzukii* is an agricultural pest on soft stone and berry fruits and the study of its olfactory system is facilitated by its taxonomic proximity to *D. melanogaster*. Understanding how host selection is mediated by the olfactory system in *D. suzukii* may help improve pest management and at the same time improve fundamental knowledge on host selection in polyphagous insects.

1.3.1 The peripheral olfactory system

D. melanogaster serves as a great model to describe the olfactory system and tackle its mechanism because it has been thoroughly described and reviewed (Renou *et al.* 2019). yet, mechanisms by which odours are encoded and processed remain to be fully understood (Vosshall 2000; Stocker 2009; Mohamed *et al.* 2019). Olfactory systems are described as relatively well conserved among Drosophilids with similar structures and chemosensory genes (Guo and Kim 2007; Keesey *et al.* 2015; Keesey *et al.* 2019). In *D. suzukii*, anatomical structures on the antenna and maxillary palp and chemosensory genes are strikingly similar to the ones of *D. melanogaster* (Ometto *et al.* 2013; Hickner *et al.* 2016; Ramasamy *et al.* 2016; Karageorgi *et al.* 2017). The olfactory system in *D. melanogaster* is

described below (Figure 1-5, Table 1-1) with emphasis on the structures dedicated to host/food odour detection.

1.3.1.1 Structural units on the peripheral olfactory system

The third antennal segment and the maxillary palp form the peripheral olfactory system in *Drosophila*. They are covered in hair and finger-like structures called sensilla and house approximately 1200 and 120 olfactory receptor neurons (ORNs) on the antenna and maxillary palps respectively (Shanbhag *et al.* 1999). In each sensillum the dendrites of one to four ORNs bath in the sensillum lymph that is secreted by supporting cells surrounding them. The cell bodies of the ORNs are located below the surface among supporting cells. Three morphologically distinct types of sensillum were described, localized in distinct yet overlapping regions on the surface: basiconic, trichoid and coeloconic sensilla (Riesgo-Escovar *et al.* 1997; Shanbhag *et al.* 1999; Couto *et al.* 2005). The function of ORNs located in these sensillum types can be determined by recording extracellularly their responses to odorant molecules (Kaissling 1995).

1.3.1.1.1 The basiconic sensilla

The basiconic sensilla described on antennae and maxillary palps contain the classes of ORNs responding to diverse food related odorant molecules such as esters, alcohols, and aldehydes (Clyne *et al.* 1997; De Bruyne *et al.* 1999; De Bruyne *et al.* 2001). Based on their morphology and the response of the ORNs they house to panels of odours, thirteen functional basiconic sensillum types have been characterized across several studies (De Bruyne *et al.* 1999; De Bruyne *et al.* 2001; Elmore *et al.* 2003; De Bruyne and Baker 2008; Dweck *et al.* 2016). ORNs are tuned to several odorants with varying affinity some of which are inducing the strongest response of one ORN class only (Table1-1) enabling a rapid identification (Hallem *et al.* 2004; Couto *et al.* 2005; Hallem and Carlson 2006; Hallem *et al.* 2006).

On the antenna, three large types (LB), 2-3.5 µm diameter and 10 µm long were described on the medial side of the funiculus (third antennal segment): the ab1, ab2 and ab3 (De Bruyne *et al.* 2001). They can be recognised by the high affinity of one of the ORNs they house to CO_2, ethyl acetate and 2-heptanone respectively. Seven small types (SB), 1.7 µm diameter and 8 µm long were described around the LB: ab4-ab10 (De Bruyne *et al.* 2001; Elmore *et al.* 2003). They can be recognised by the high affinity of one of the ORNs they house to (*E*)-2-hexenal, pentyl acetate, (*RS*)-1-octen-3-ol, 6-methyl-5-hepten-2-one, ethyl butanoate, acetophenone and 2-phenyl ethanol respectively. Three types of thin basiconic (TB) were described on the maxillary palp: pb1-pb3 (De Bruyne *et al.* 1999; Dweck *et al.* 2016). They can be recognised by the response of one of the ORNs they house to ethyl

acetate, fenchone and 2-heptanone respectively. Lastly, two additional types were described on the antenna called ab11 and ab12 (Kwon *et al.* 2010) but not fully characterised. All but the ab1, ab11 and ab12 types contain the dendrites of two ORNs (Riesgo-Escovar *et al.* 1997; Shanbhag *et al.* 1999; Kwon *et al.* 2010). The ab1 house four ORNs, ab11 and ab12 house each three ORNs.

1.3.1.1.2 Other sensillum types

Approximately 100 coeloconic sensilla on the antenna, with an extra 40 within the sacculus, are organised in four functional types, ac1-ac4 (Shanbhag *et al.* 1999; Yao *et al.* 2005). They are short thin structures, about 4 µm long, and house 1-3 ORNs, which respond to food components (amino acids, acids) and to humidity. Lastly, approximately 100 trichoid sensilla from four different types (at1-at4) are segregated by type on different regions of the antenna (Shanbhag *et al.* 1999; Carlsson and Hansson 2002; Lin and Potter 2015). They are hair like structures approximately 20 µm long and contain 1-3 neurons. They contain neurons known to detect volatile pheromones. For instance, the response of the antennal trichoid type 1 (at1) to cis vaccenyl acetate has been particularly well-studied (van der Goes van Naters and Carlson 2007; Datta *et al.* 2008; Dekker *et al.* 2015). Intermediates ai1, ai2 and ia3 were identified on the antero-lateral side (Lin and Potter 2015). A recent study determined that only ORNs in basiconic sensilla responded to fruit volatiles. Exceptions are for the intermediate type ai2. One of its ORNs responds to fruit volatiles, the terpenes limonene and valencene (Dweck *et al.* 2013; Dweck *et al.* 2018). In addition to responding to food odours (see above) one of the ORNs in the ab9 responded to (*Z*)-4-undecenal, a fly cuticular hydrocarbon (Lebreton *et al.* 2017). Lastly, the ab1 house one ORN tuned to carbon dioxide (Jones *et al.* 2007).

1.3.1.2 The detection of odorant molecules

Volatiles in the air around the sensilla diffuse through pores on the sensillum wall into the sensillum lymph (Kaissling 2001) and interact with odorant binding proteins (OBPs). OBPs are a class of extracellular proteins secreted by supporting cells of the sensillum. They are thought to act as transporters of the lipophilic odorant molecules in the more hydrophilic sensillum lymph (Zhou *et al.* 2004; Pelosi *et al.* 2006; Leal 2013; Larter *et al.* 2016). Their inhibition also induced impairments in the detection of odorant molecules by ORNs in basiconic sensilla and changes in behavioural responses (Swarup *et al.* 2011).

1.3.1.2.1 The OR-Orco complex

Odorant molecules interact with olfactory receptors (ORs) located on the dendrites of the ORNs. Except for the gustatory receptor (GR) Gr21a located on the ab1 housed ORN ab1C,

only ORs have been described associated with ORNs in basiconic sensilla (Kwon *et al.* 2007; Vosshall and Stocker 2007; Ai *et al.* 2013).

ORs are seven trans-membrane-domain proteins forming a homo-tetrameric ligand-gated ion channel, with a co-receptor: Or83b, so-called Orco in *Drosophila* (Clyne *et al.* 1999; Larsson *et al.* 2004; Sato *et al.* 2008; Wicher *et al.* 2008; Butterwick *et al.* 2018). The four subunits comprise most likely two ORs and two Orco (but other arrangements may be possible). The binding of odorant molecules to the OR-Orco complex induces changes in the dendritic membrane potential. The membrane depolarization caused by opening of voltage-gated ion channels generate action potentials. The trains of action potentials along the axons form the sensory information that is relayed to the central olfactory system.

A family of 60 *Or genes* have been identified in *D. melanogaster* (Clyne *et al.* 1999; Vosshall *et al.* 1999). *Or* sequences are widely divergent suggesting their fine specialisation (Butterwick *et al.* 2018). Generally only one OR is expressed in one ORN but two functional ORs can be also found on one ORN (Mombaerts 2004; Goldman *et al.* 2005). Thirteen of these ORs are expressed in both the adult, and the dorsal organ of the larvae (Couto *et al.* 2005; Fishilevich and Vosshall 2005; Kreher *et al.* 2005). *Orco* is expressed in most ORNs and its sequence is highly conserved across insect species (Larsson *et al.* 2004; Hansson and Stensmyr 2011).

1.3.1.2.2 Odour specificity

The isolation and expression of individual *Or* genes coupled with extracellular recordings on ORNs and calcium imaging permitted to study the odour specificity of ORs. This was first done with Or22a and Or22b that are located on the dendritic membrane of the so called ab3A neurons in the ab3 sensilla in *D. melanogaster* (Dobritsa *et al.* 2003). After expressing a single OR (e.g. Or22a) in an “empty neuron”, responses to several odours could be recorded. The response of ab3A to the odour ethyl butanoate and several other odorants could then be attributed to Or22a. Following this approach almost all identified ORs were associated with ORNs and high affinity odours (Table 1-1).

Some ORs are narrowly tuned to few chemicals of similar molecular structure while other ORs are broadly tuned to many chemicals of different molecular structures (Hallem *et al.* 2004; Hallem and Carlson 2006; Dweck *et al.* 2016; Grabe *et al.* 2016). This way, some ORNs may be tuned to many hosts and non-host volatiles while others may respond to a few host or non-host specific chemicals.

The ORs can be highly sensitive to low doses of chemicals. For instance, less than 10 molecules hitting the antenna per second was enough to induce an antenna response in

Lepidoptera. Notably in *Bombyx mori,* a single pheromone molecule induced trains of five action potentials in one trichoid ORN (Desogus *et al.* 2003; Kaissling 2017; Kaissling 2019). ORN responses to ligands can be described by dose-response relationships following the Hill function (Lancet *et al.* 1993; Si *et al.* 2019). ORNs are spontaneously active, and odorants can either excite or inhibit the neuron's activity. The activation rate increases exponentially with the dose of chemical detected until ORNs reaches a maximal activation, so the number of spikes no longer increase with concentration. Instead, the neurons stay in a state of high firing rate several seconds after the stimulus, i.e. the time to return to the spontaneous activity level increases (De Bruyne *et al.* 1999).

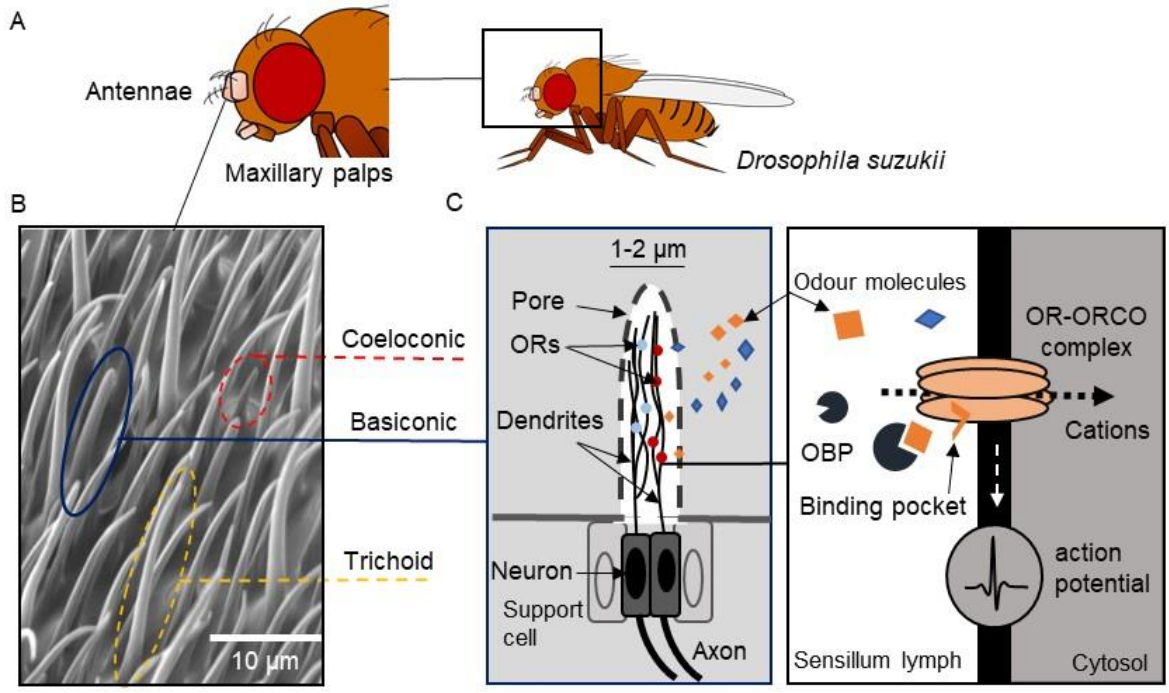


Figure 1-5 A brief overview of the olfactory system in Drosophilids

A) Schematic *of D. suzukii* showing the peripheral olfactory system: antennae and maxillary palps. B) olfactory receptor neurons (ORNs) are located on the surface of the antenna and maxillary palps in functional sensillum types. Three types of sensillum are visible with scanning electron microscopy (photograph by Rebecca Lauder, Rothamsted Research) and light microscopy: they house 1-4 ORNs each that are tuned to odours from conspecifics (trichoids, dotted yellow line), amines (ceoloconics, dotted red line) and host/food (basiconics, plain blue line). B) Schematic of a basiconic sensillum with two ORNs. C) Odour molecules diffuse through pores and then reach the olfactory receptors (ORs) perhaps helped with odorant binding proteins (OBPs). Located on the dendritic membrane, ORs are part of a complex with ORCO. Flux of cations (only one way shown) induce de- and re-polarisation of the dendritic membrane (measured in the form of action potentials). Trains of action potentials are generated and then propagate down the axons towards glomeruli in the antennal lobe. Schematics based on descriptions from Shanbhag *et al.* (1999), Pelosi *et al*. (2006), Butterwick *et al.* (2018).

1.3.2 Transmission of information towards the central olfactory system

1.3.2.1 Olfactory circuitry

ORNs project from the antenna and maxillary palp onto the antennal lobe constituted of approximately 50 distinct glomeruli (Stocker 1994; Laissue *et al.* 1999; Vosshall 2000; Laissue and Vosshall 2008). The ORNs expressing the same ORs converge into the same glomeruli (Laissue and Vosshall, 2008). From the glomeruli the ORN synapses are connected to second-order olfactory neurons projecting towards the olfactory cortex (comprising of the mushroom bodies and lateral horn). Local interneurons (LNs) connect glomeruli with each other and mediate excitation and inhibition (Silbering and Galizia 2007). Each glomerulus is served by a distinct class of Projection Neurons (PNs) relaying the inputs from the antennal lobe to the Kenyon cells of the mushroom bodies (Marin *et al.* 2002) where learning and memory formation of odour stimuli takes place in *Drosophila* (Heisenberg 2003; Turner *et al.* 2008; Aso *et al.* 2014). Each glomerulus appears also to project to localised regions in the lateral horn (Marin *et al.* 2002; Jefferis *et al.* 2007).The processing of information at each stage is still under investigation (Stocker 2009; Grabe *et al.* 2016). Lastly, the glia covers and interact with the existing neuronal networks. Its role in modulation of neuronal transmission of signals, learning and memory is studied and reviewed in *Drosophila* (Parker and Auld 2006; Malik *et al.* 2013; Kremer *et al.* 2017).

1.3.2.2 Quantification of sensory information

Olfactory responses by ORNs in *Drosophila* can be initiated within milliseconds of odour reaching the antenna (Egea-Weiss *et al.* 2018). ORNs encode odours via increase/ decrease and latencies in the spontaneous firing rate of action potentials (Clyne *et al.* 1997; De Bruyne *et al.* 1999; Egea-Weiss *et al.* 2018). Generally, the firing rate is positively correlated with the concentration of an odorant molecule. A compound of low affinity in higher dose may thus induce a similar firing rate than a compound of high affinity in a lower dose. Certain odorants also elicit a decrease in the firing frequency of some ORNs while eliciting an increase in other ORNs, a process called inhibition (de Bruyne *et al.* 2001).

It is unclear how the olfactory system deals with timing and intensity of odorant detection but recent studies found that the timing and latency of spiking activity depend on the identity of the odorant (Egea-Weiss *et al.* 2018). The number of spikes following onset of stimulus is characterised as a quantifiable variable for the intensity of response. It is used to identify which and how ORNs respond to various odours in most olfactory studies. A different angle of research started to emerge to look at the processing from ORNs all together in addition to each single ORN response. For instance, ORNs can adapt to both the average intensity

and the variance in intensity of odour signals by means of interactions between olfactory units (van der Goes van Naters 2013; Gorur-Shandilya *et al.* 2017; Si *et al.* 2019).

1.3.2.3 Processing of the output from ORNs

The classes of ORNs may be activated with a certain intensity or firing rate to induce a behavioural response. Such threshold of activation for each class of ORNs has not been characterised. Behavioural responses may also be "triggered" via a threshold level given by the total summation of all olfactory input (all classes of ORNs included) into specific glomeruli. Examples of this theory were found in moths and flies. The AL can relay a summed outcome of the separate signals received from the ORNs in moths (Kuebler *et al.* 2012). In *Drosophila* larvae, Kreher *et al.* (2008) correlated the sum of the action potentials from all the 21 larval receptors with the behavioural responses elicited by a panel of odorant molecules.

Other properties of action potential responses are also difficult to assess. For instance, after the start of a stimulus pulse the generation rate of action potentials of an ORN may increase rapidly to reach a maximum and then either remain at a plateau or decay during the stimulus, whilst after stimulus offset the response may decay rapidly or slowly (De Bruyne *et al.* 1999). Rapid decay is often followed by a reduced rate before non-stimulated activity is restored to pre-stimulus levels. Different kinetic response properties depend on the OR and on the stimulus. The behavioural consequences of the kinetics are poorly understood. Tonic responses that are maintained beyond stimulus offset have been hypothesised to provide a peripheral memory mechanism, whereby the insect is informed about recent stimulus history (Boyle *et al.* 2016).

Table 1-1 Association of ORNs, ORs and OBP and evolutionary events shaping the OR repertoire in D. suzukii

Associations of olfactory receptor neurons (ORNs), odorant binding proteins (OBPs), olfactory receptor (ORs) and glomeruli as described in *D. melanogaster* (Laissue *et al.* 1999; Laissue and Vosshall 2008; Kwon *et al.* 2010; Swarup *et al.* 2011; Dweck *et al.* 2013; Leal 2013; Ometto *et al.* 2013; Larter *et al.* 2016). Kwon *et al.* (2010) described the ab11 and ab12. Dweck *et al.* (2013) described the AI1-3. Only the OBPs which genes were found in female *D. suzukii* are reported. Their presence was confirmed by comparison of sequences between *D. suzukii* and *D. melanogaster* from the Swissprot database (data not shown) with guidance from J-J. Zhou (Rothamsted Research).

ORN	OBP ¥	OR	Glomerulus	Key ligand*	In *D. suzukii*
ab1A	83a, 83b, 28a	Or42b	DM1	Ethyl acetate	
ab1B		Or92a	VA2	2,3-Butanedione	
ab1C		Gr21a	V	Carbon dioxide	
ab1D		Or10a	DL1	Methyl salicylate	
ab2A	83a, 83b, 28a	Or59b	DM4	Ethyl acetate	
ab2B		Or85a	DM5	Ethyl butanoate	Loss of function
ab2B		Or33b	DM3 + DM5	Isoamyl acetate	
ab3A	83a, 83b, 28a, 19a	Or22a	DM2	Ethyl butanoate	Loss of function[2]
ab3A		Or22b	DM2		Duplicated[2]
ab3B		Or85b	VM5d	2-Heptanone	
ab4A	28a, 19a	Or7a	DL5	(*E*)-2-Hexenal	
ab4B		Or56a	DA2		
ab5A	28a, 19a	Or82a	VA6	Geranyl acetate	
ab5B		Or47a	DM3	Pentyl acetate	
ab5B		Or33b	DM3 + DM5		
ab6A	28a, 19a	Or13a	DC2	(*RS*)-1-Octen-3-ol	
ab6B		Or98b	VM5d		Loss
ab6B		Or49b	VA5		
ab7A	83a, 83b, 28a, 19a	Or98a	VM5v	Sulcatone*[1]	Loss
ab7A		Or67c	VC4	Ethyl lactate	
ab7B					
ab8A	28a	Or43b	VM2	Ethyl butanoate	
ab8B		Or9a	VM3	2,3-Butanedione	Positive selection
ab9A	28a	Or67b, Or69aA	VA3		

ab9B		Or69aB	D		2 splices became 4-7
ab10A	83a, 83b, 28a, 19a	Or67a	DM6	2-Phenylethanol	Quadruplicated + 1 pseudogen[1], Duplicated[2]
ab10B		Or49a	DL4		Duplicated[2]
ab10B		Or85f	DL4		
pb1A		Or42a	VM7	Ethyl acetate	Expanded[1], 2 isoforms[2]
pb1B		Or71a	VC2	4-Methylphenol	
pb2A		Or33c	VC1	Fenchone	
pb2A		Or85e	DM5	Ethyl acetate	
pb2B		Or46aA	VA7I	4-Methylphenol	2 splices, 80% conserved
pb3A		Or59c	1		Duplicated[2]
pb3B		Or85d	VA4	2-Heptanone	
ai2A		Or19a		Valencene	Duplicated[2]
ai2B		Or83c			
ai2C		Or23a			Quadruplicated
ai3A		Or2a			Positive selection
ai3B		Or43a			
ab11A					
ab11B					
ab11C					
ab12A					
ab12B					
ab12C					

[¥]OBPs associated with basiconic sensillum types but not specific to the ORN/ ORs (Swarup *et al.* 2011). *Key compounds are ligands for which one of the ORs have a very high affinity enabling identification of the sensillum types. *[1]6-Methyl-5-hepten-2-one. Evolutionary events in the *Suzukii* lineage within the Sophophora group from Hikner *et al.* 2016 ([1]) and Ramasamy *et al.* 2016 ([2]). Unless stated otherwise, both referred the events.

1.3.3 Encoding of odours

Each host plant may be represented by a balanced mixture of host specific and ubiquitous compounds they release (Bruce *et al.* 2005). These host plant signals also depend on the physiological state of the plant (e.g. stress may cause the release of different volatiles) (Heil 2014). The detection of host plants by the olfactory system of the insect is also influenced

by the physiological state of the insect at a given moment. For instance, mating status or starvation were shown to influence the sensitivity of classes of ORNs to chemicals (Martel *et al.* 2009). As presented above (section 1.2.2), the sensory representation from individual components may differ from the mixture (Bruce and Pickett 2011). For instance, a blend of chemicals (e.g. the whole host odour bouquet) may activate or inhibit additional classes of ORNs compared to individual components hereby providing the insect with different information. This way the response to a mixture may not be predicted from the response to the individual components (Laing *et al.* 1984). Competition and antagonist/synergist effects of chemicals can be seen at the level of single ORN (De Jong and Visser 1988; Carlsson and Hansson 2002) and from interactions between co-localised ORNs (Su *et al.* 2012; van der Goes van Naters 2013).

1.3.3.1 Tuning of ORs

When insects are exposed to a multitude of cues, the olfactory system must be able to process them simultaneously. Insects generally must recognise relevant information from a background of many chemicals as host plants are rarely in isolation. Using one chemical or blend of chemicals that is common to multiple hosts may be dangerous for polyphagous insects since many ubiquitous compounds are shared between plant species and may also be produced by predators. They may be attracted to the wrong place if additional cues that are specific to hosts are not included. For instance, enantiomers of nepetalactol are components of the parasitoid wasp *Leptopilina sp.* body odour (unknown isomer), an aphid sex pheromone and are produced by catnip plants (Dawson *et al.* 1987; Zhu *et al.* 2011; Ebrahim *et al.* 2015).

1.3.3.2 Classes of ORNs have dedicated roles

Subsets of ORNs that are dedicated to attraction or avoidance were first described by Hansson *et al.* (1999). Notably, the intraspecific communication (i.e. sexual communication), predatory avoidance and host selection in strict specialists (i.e. which recognise and accept a very small subset of taxonomically related plant species) were shown to be mediated by the activation of one ORN class. This falls under one of the theories of how encoding of host odours induce behaviour: that one or a few ORNs activated would induce a specific behaviour thereby showing some behaviours are served by a dedicated olfactory circuitry (Hansson *et al.* 1999; Hansson and Christensen 1999; Suh 2004; Kwon *et al.* 2010; Lebreton *et al.* 2017). Additional supporting studies looked at the tuning of ORNs and glomeruli and their specificity. They highlighted that glomeruli and their innervating glomeruli were also associated with specific behaviours (also called the valence) and with ecological function (i.e. mates, food sources). Glomeruli were additionally

activated by specific chemical classes, as for instance esters and aldehydes activated distinct glomeruli (Grabe and Sachse 2018).

1.3.3.3 Classes of ORNs form a combinatorial pattern

Recent data suggests that the identity, number of ORNs, the intensity and timing of activation form a unique combinatorial pattern on the peripheral olfactory system and that the outcome signals are modulated by interactions with other olfactory units (Hallem and Carlson 2006; Haverkamp *et al.* 2018; Mohamed *et al.* 2019). At several stages of the olfactory circuitry (between the first detection by ORNs and the final stage that is the behavioural response) all units of the olfactory system may contribute to modulate the information received from a combination of ORNs. This hypothesis states that i) a combination of several classes of ORNs are simultaneously activated; ii) the intensity and the timing of these activations form unique patterns; iii) these patterns are also modulated by cross talks between co-localised ORNs and between glomeruli (Egea-Weiss *et al.* 2018; Mohamed *et al.* 2019; Si *et al.* 2019).

1.3.3.4 Encoding of complex host odours

Specialist feeders appear to detect host specific cues that are encoded by dedicated single neuron pathways, mediating attraction (del Campo and Miles 2003; McBride 2007; Auer *et al.* 2019). However, in more generalist species it remains relatively complicated to determine how host selection happens when taxonomically diverse hosts can be detected and recognised as suitable.

Olfactory detection of host fruits may be a mechanism somewhat in between of extreme hypotheses (Figure 1-6). All hosts release shared and specific volatiles that are detected by the fly (Figure 1-6, A). The fly may detect (one or many) chemicals that are shared by all hosts enabling them to distinguish hosts from non-suitable fruits (i). Alternatively, (one or many) specific chemicals are detected thereby allowing discrimination of each host type (ii). Host selection can also be considered by how the olfactory system processes host cues (Figure 1-6, B). Chemicals forming the detected fruit headspace ((i) or (ii)) are encoded by classes of ORNs on the peripheral olfactory system. Hosts may thus be recognised because headspaces from hosts all activate a common subset of ORNs that is different from non-suitable fruits (Hypothesis 1), or each host fruit headspace activates a specific subset of ORNs thereby enabling discrimination of each fruit type (Hypothesis 2).

It remains unclear how a species which can sustain a larger range of hosts (whose commonality is unclear) recognise them from non-suitable fruits. Overall, olfactory and visual stimuli, the memory of stimuli encountered and the physiological state of the individual all are processed together and enable behavioural responses. How host selection

is mediated by encoding of fruit odours by the peripheral olfactory system in the polyphagous fly *D. suzukii* is addressed in this thesis.

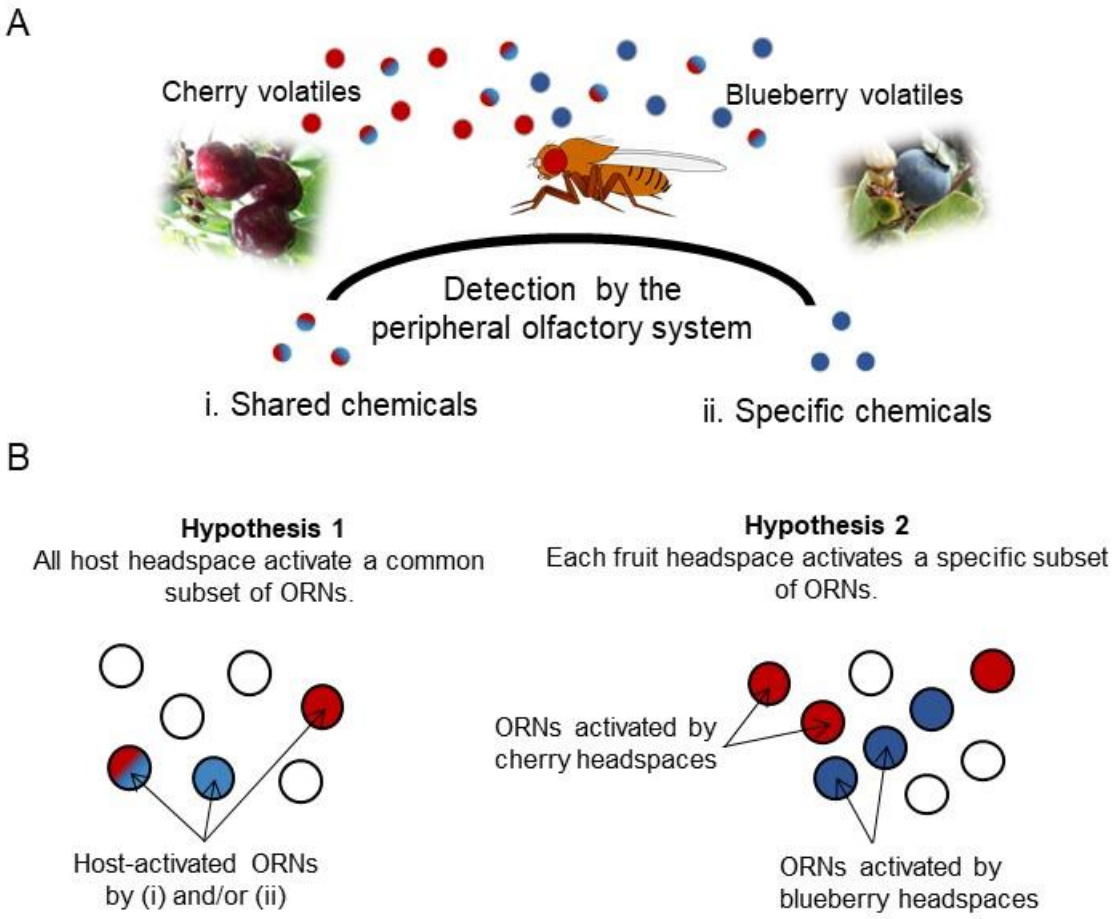


Figure 1-6 Hypothesis on the encoding of fruit odours mediating host selection in D. suzukii

A) All hosts may release shared and specific volatiles that are detected by the fly. This is illustrated with two extreme scenarios: flies may detect volatiles shared by all hosts (one or many chemicals) so they can recognise a host fruit; ii) flies may detect one or a blend of specific chemicals so they can discriminate between host type. Dots illustrate odorant molecules from cherry (red), blueberry (blue) and from both (red/blue). B) Host selection can also be considered by how the peripheral olfactory system process host cues. Chemicals activate classes of olfactory receptor neurons (ORNs) on the peripheral olfactory system. Hosts may thus be recognised by patterns of activation in between of two extreme hypothesis: 1) all host headspaces (specific and/or shared chemicals) activate a common subset of ORNs, enabling the flies to recognise hosts from non-hosts; 2) each fruit headspace activates a specific subset of ORNs, enabling the flies to discriminate between host type. Filled circles illustrate classes of ORNs that can be

activated by odorant molecules from blueberry (blue), cherry (red) and from both (blue/red). Based on Bruce and Pickett (2011).

1.4 PRESENTATION OF THE THESIS

1.4.1 Summary

D. suzukii is responsible for major economic losses in fruit farming because females lay eggs in undamaged ripening fruits which otherwise would be available for retail. They detect plant volatiles with their peripheral olfactory system and the subsequent central processing mediates host selection for oviposition, mating and/or feeding. Understanding how their highly sensitive system has adapted to successful polyphagy would provide fundamental knowledge on insect-host interactions. The understanding of *D. suzukii*-host interactions would identify mechanisms that induce attraction and allows novel semiochemicals to be developed into lures for management of the fly in commercial crops.

The specialisation of many insect species on a single plant family or clade often involves an adaptation to detect one or a small number of host specific chemicals. They can recognise these host specific cues from a background of chemicals released from many plants and animals from the environment. By contrast, knowledge on host selection by polyphagous insects is limited because specific cues and shared cues from their taxonomically diverse hosts have yet to be identified. *D. suzukii* may have adapted to many host types via increased tolerance to multiple plant substrates. Its olfactory system may thus enable the fly to discriminate and rank its many hosts enabling the switch from one to another during the year. Alternatively, host choice in *D. suzukii* may result from a specialisation on common characteristics shared by all hosts such as the yeast it was associated with, as is the case in other *Drosophila sp.* (see above).

An innovative approach is to look at how multiple host odours are encoded by the peripheral olfactory system in polyphagous insects. ORNs are activated by odour bouquets from taxonomically diverse attractive hosts. Are attractions mediated by a single dedicated olfactory circuit (one or perhaps a combination of classes of ORNs) enabling discrimination of host from non-suitable fruits? Does each host activate a specific olfactory circuit (one or a combination of several ORNs) enabling discrimination among various attractive substrates?

1.4.2 Aim and hypothesis

The aim of this research was to understand the mechanisms that underlie detection and discrimination of fruits by the peripheral olfactory system and allow the polyphagous fly *D. suzukii* to select hosts. A comparative study was also made with *D. melanogaster*, a specialist feeder on yeast, found on many damaged and overripe fruits. The study focused on the encoding of ripe fruit odorant bouquets by the ORNs in basiconic sensilla of the adult gravid female. This is the first study which focuses on the encoding of the whole fruit bouquet in addition to individual constituents. It is also the first study using whole fruits that have not been cut or macerated. The objective of this approach was to mimic the ecologically relevant fruit odours that are detected and used for host selection in *D. suzukii*.

The hypothesis tested is that chemically diverse odour bouquets are encoded by specific and overlapping subsets of ORNs on the peripheral olfactory system of the fly thereby allowing gravid females *D. suzukii* to recognise taxonomically diverse fruits suitable for oviposition.

A common pattern of activation of ORNs by taxonomically diverse or shared fruit volatiles enables the fly to select host from non-suitable fruits rapidly before landing on the fruit thereby optimising host selection. In addition, fruit-specific patterns of activation of ORNs enables females to discriminate between hosts thereby developing preferences for the most suitable host fruit from taxonomically diverse plant species and from closely related cultivars within plant species.

1.4.3 Presentation of the chapters

In mated females *D. suzukii*, ripe fruit odours are encoded by specific and overlapping subsets from seven classes of ORNs on the peripheral olfactory system enabling recognition of taxonomically diverse host and non-suitable fruits (Chapter 3). Fruit headspaces form antennally active bouquets of mostly fruit-specific chemicals. They activate specific and overlapping classes of ORNs from the seven that responded to the whole headspaces. The encoding of odours by combinations of these subsets mediate behavioural responses similar to the fruits (Chapter 4). Furthermore, the development and improvement of control techniques for *D. suzukii* may be addressed with the idea of a model of how the encoding of odours by the olfactory system mediates behaviours: it is possible to identify attractive or repelling odours based on which ORNs they activate. The ripening fruit volatiles identified are candidate tools for management programs (Chapter 4).

An additional study on several grape cultivars supports the neural model as results showed that the same classes of ORNs mediating discrimination among taxonomically diverse fruits

also mediates discrimination of grape cultivars that are host and non-suitable for oviposition (Chapter 5). This neural model mediates the detection and selection of taxonomically diverse hosts and closely related hosts belonging to the same species in *D. suzukii*.

A comparative study with *D. melanogaster* demonstrated that the model of olfactory detection of ripening fruits by dedicated classes of ORNs was maintained in two closely related *Drosophilids*. Functional changes associated with an olfactory specialization on ripening fruits in *D. suzukii* were identified (Chapter 6).

Lastly, an impairment of the detection of a foliage associated volatile β-cyclocitral, via the ab3A neurons was discovered. As this class of ORNs was reported to be important for host specialization in *Drosophilid*s, the effects of this impairment on ripe fruit detection were described and its origins discussed (Chapter 7).

The results presented in the chapters support the hypothesis presented. Neuronal models of olfaction regarding host detection in polyphagous insects are discussed in the respective chapters.

2 Materials and Methods

First description of *Drosophila suzukii*, originally called *Leucophenga suzukii* by Dr Shounen Matsumura. 日本昆虫大図鑑, Page 214, Matsumura, 1931. Copyright © 2011 National Diet Library.

2 MATERIALS AND METHODS

2.1 INSECTS

D. suzukii larvae and pupae were obtained from Oxitec Ltd (originating from the San Diego *Drosophila* Stock Centre, UCSD, USA). Two populations were created and were reared separately, at Cardiff University and at Rothamsted Research.

In addition, *D. suzukii* larvae and pupae were collected from wild raspberry in the UK and by W. Van der Goes van Naters in The Netherlands in 2017. Adults from field collected populations in Italy in 2018 were kindly provided by Alberto Grassi (FEM, Italy). *Drosophila melanogaster* larvae and pupae originated from an Oregon R strain (Bloomington Stock Centre, USA).

Flies were kept at room temperature (20 °C), with a 12:12 light: dark cycle. Flies used in all experiments were reared on a media containing agar (10 g/l), cornmeal (25 g/l), sugar (41 g/l), inactive yeast (14 g/l), propionic acid and 10 % (in ethanol) nipagin (4 ml/l). For *D. suzukii*, approximately every two generations food was supplemented with a piece of fresh fruit (e.g. raspberry, blueberry) to provide variation in their dietary intake. Adults were collected on the day of emergence and aged in mixed sex vials for 5-8 days. Adult gravid females were used, unless otherwise indicated. Tests were done at room temperature (22-24 °C), with a relative humidity between 40 and 60 %.

Four distinct rearing of *D. suzukii* (from the laboratory population) were created in order to test the role of host exposure in host preference (Chapter 3). Media was implemented with fresh fruit pieces, of either strawberry, raspberry, blueberry and grape. Unfortunately, the rearing on strawberry was lost after several generations. Each group was kept exclusively on one fruit type for over 12 generations.

2.2 FRUITS

Harvested ripe fruits from several cultivars of strawberry (*Fragaria ananassa*), raspberry (*Rubus idaeus*), blueberry (*Vaccinium corymbosum*), grape (*Vitis vinifera*), orange (*Citrus sinensis*) and tomato (*Solanum lycopersicum*) were used on the day and second day after purchase from local shops for headspace collection, electrophysiological recordings and behavioural assays (Table 2-1). Fruits were kept at room temperature at least one hour before use. Stems and basal leaves still attached to the fruit were kept if their removal could cause damage to the fruit (strawberry, grape and tomato) which could alter the volatile

profile. For headspace volatile collection and electrophysiological recordings 100-120 g of strawberry, blueberry and grape, and 150-200 g of orange and tomato were used.

Females may be responsive to plant volatiles that are associated with ripening fruits on the plant and which may not be released by harvested fruits. In order to identify these, all fruit plants but grape vines were grown in greenhouse for one year. The aim of the procedure was to collect volatiles from non-harvested fruits and from the leaves of plants bearing fruits. The following plants were grown by Jill Maple and her team at Rothamsted Research: Strawberry (cv. Elsanta), raspberry (cv. Unknown), blueberry (cv. Liberty.), orange (cv. Valencia) and tomato (cv. Mecano).

Due to the limited availability of fruits in retail, several cultivars had to be used. A separate experiment was designed to assess the neuron response patterns to headspaces of fruits from several cultivars to determine whether females would be able to olfactory discriminate between cultivars of a same host species. The electrophysiological responses to three cultivars of blueberry (cv. Biloxi, Ventura and Legacy) and strawberry (cv. Sabrina, Winter Star and Cuna) were measured.

The electrophysiological and behavioural responses to five cultivars of grape (*Vitis vinifera*), which differed by their susceptibility to be oviposited by *D. suzukii* was assessed. Cultivars of wine grape were collected in Italy in the late summer 2017, kindly hosted by Florian Sinn (Südtiroler Beratungsring, Italy). Clusters of ripe Schiava, Traminer, Merlot, Pinot Noir and Lagrein cultivars were harvested and used in behavioural assays and headspace volatile collection.

Table 2-1 Fruits used for headspace volatile collection and electrophysiological recordings

Fruit	Species	Family (Clade)	Cultivar
Strawberry	*Fragaria ananassa*	Rosaceae (Eurosid I)	Elsanta* ** Sabrina Winterstar Cuna Malling Centenary
Raspberry	*Rubus idaeus*	Rosaceae (Eurosid I)	Radiance* Maravilla Adelita
Blueberry	*Vaccinium corymbosum*	Ericaceae (Asterids)	Jewel* Liberty** Brigitta Ventura Legacy Biloxi
Grape	*Vitis vinifera*	Vitaceae	Sugraone*

		(Core Eudicots)	thompson Crimson Flame
Orange	*Citrus sinensis*	Rutaceae (Eurosid II)	Valencia* ** Lane-Late Sajustjana Midknight Navelina
Tomato	*Solanum lycopersicum*	Solanaceae (Euasterids I)	Mecano* Roterno

*Cultivars used for harvested fruits headspace collection. **Cultivars grown for whole plant headspace collection. Cultivars used depended on retail availability.

2.3 CHEMICALS

2.3.1 Odour cartridges for electrophysiological recordings

To record the electrophysiological responses to fruit headspaces and volatiles, the odour stimuli were delivered using odour cartridges. These were Pasteur pipettes holding chemicals. Stimuli were presented by passing a pulse of air through the Pasteur pipette into a carbon-filtered humidified air stream directed at the fly preparation.

An aliquot of 30 µl of a solution of a chemical in paraffin oil was deposited on a filter paper (15 mm diameter, Whatman grade 1, USA) and placed within the larger end of a glass Pasteur pipette (Thermo Fisher Scientific, USA) which was then closed with a 1 ml pipette tip. Dilutions of chemical solutions used ranged from 10^{-4} µl/ml to 10^{-9} µl/ml in decadic steps. Exhaled air (breath) was used as a stimulus to test the response to CO_2. Each odour cartridge was used for a maximum of four stimuli.

To use fruit headspaces as a stimulus, 100-180 g of undamaged fruits were placed in a 1 l glass beaker covered with aluminium foil. Air from the beakers was sucked with a 20 ml plastic syringe to fill a glass Pasteur pipette (cartridge, as above) before delivering each stimulus. An empty beaker served as control (ambient air). A similar technique was used to collect headspaces above baits in behavioural assays.

2.3.2 Chemical baits for behavioural assays

Baits with dispensers were created to test the responses to chemical stimuli in behavioural assays. The dispensers were chosen because they enabled to produce a constant release rate of chemicals during behavioural assays lasting five hours (Appendix 1). Chemicals

were loaded on three types of dispensers from which they diffused into the air: glass capillaries, rubber septa and filter papers.

In 4-choice cage assays (see below), glass capillaries were used as dispensers in baits. Glass capillaries enabled to control the release rate of individual chemicals. Indeed, different sizes of capillaries permitted to set the surface of contract between the chemical and the air in order to achieve a desired release rate (Pers. Comm. Keith Chamberlain, Rothamsted Research). Capillaries were made of a 30 mm long piece of glass capillary with one end heat-sealed. Two different diameters were used made from 100 µl capillaries intraMARK and intraEND (Blaubrand, Germany). They were filled to the top with approximately 30 µl and 50 µl respectively of solution before each trial. Solution were 10^{-4} µl/ml (1% v/v) dilutions in paraffin oil, unless otherwise stated.

In wind tunnel assays (see below) the chemical baits were created using rubber septa (unknown supplier, Rothamsted research) and filter paper (Whatman Grade 1). 100 µg (or 10 µl of a 1% v/v solution was loaded on a rubber septum (in hexane) or on a filter paper (in paraffin oil). Chemicals were loaded and left a few minutes before testing to allow the hexane to evaporate.

2.3.3 Authentic standards

All chemicals that could exist as stereoisomers were used in racemic mixture unless otherwise indicated and were of the highest purity available on purchase (Sigma-Aldrich Inc., USA and TCI Tokyo Ltd., UK). (*Z*)-3-Hexenal was in a 50:50 solution with the stabiliser triacetin. Isopropyl pentanoate, ethyl-3-methyl-2-butenoate and (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol were synthetized by David Withall (Rothamsted Research).

(*S*)-Linalool was purified from an existing extract. Purification was done through a flash column. The neat compound was diluted into a chromatography solvent made of 8 % ethyl acetate into petroleum ether. It ran on a thin layer chromatography silica plate using the same solvent as carrier and migrated with a Ratio Rf = 0.36. The diluted compound was then run through a flash column made of silica 60 and the same chromatography solvent used above. The fractions containing (*S*)-linalool were collected and the solvent evaporated using vapo-rotation and vacuum.

The identity, purity and chirality of the obtained compound were verified with coupled gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance spectroscopy (NMR) and polarimetry.

2.4 FRUIT HEADSPACE COLLECTION

Chemicals released from fruits (headspaces) were collected by dynamic headspace collection (DHC) (Figure 2-1). The headspace extracts were used for electrophysiological recordings and analytical chemistry to identify which fruit volatiles were detected by gravid female *D. suzukii*.

2.4.1 From harvested fruits

Headspace collections were performed as follow. Fruits were placed under a glass chamber closed with two semi-circular aluminium plates with a filter paper (Whatman 1001-150) underneath the fruits. Charcoal filtered air was pushed in via an inlet port at a rate of 600 ml/min. Air was sucked out via an outlet port at a rate of 500 ml/min, creating a positive pressure in the chamber. This positive pressure ensured that no contamination from outside air would enter the system. The air outlet fed through a glass tube containing 50 mg Porapak Q polymer (mesh size 50/80, Supelco) which absorbed the chemicals, held between two plugs of glass wool. The airflows were controlled by air pumps (Leighton Buzzard, UK). All connections were made from PTFE tubing and ferrules. Between collections, glassware and aluminium plates were washed with detergent, rinsed with distilled water and 100% acetone, then baked at 180 °C for at least 2 h. Three replicates of each fruit and one control (empty chamber) were simultaneously collected during 24 h.

2.4.2 From whole plants

To collect headspaces from fruits still attached on the plant, DHC were done as follow. Plant materials were held in a roasting bag (Sainsbury, UK). Charcoal filtered air was pushed in via an inlet port at a rate of 600 ml/min. Air was sucked out via an outlet port at a rate of 500 ml/min, creating a positive pressure in the chamber. The outlet air passed through a 50 mg Tenax TA polymer (mesh size 60-80, Supelco) which absorbs the chemicals, held between two plugs of glass wool. All other parameters were as described above.

2.4.3 Headspace volatile extracts

Chemicals retained in the Porapak Q following the DHC were eluted with 750 µl re-distilled diethyl ether into a glass vial. Diethyl ether was redistilled to ensure purity and removal of the stabiliser (the antioxidant butylated hydroxytoluene) which eluting peak (see below) may overlap and mask eluting peaks of interests on gas chromatography. The extracts were reduced to approximately 100 µl under a gentle stream of nitrogen. The concentration procedure was followed to allow the detection of compounds that are released in very low

amounts over the 24 h collection. The disadvantage is that highly volatile compounds may evaporate along with the solvent which would reduce their representation in the extract.

Success and consistency of collections were verified by gas chromatography. Aliquots of 4 µl were injected in a gas chromatograph (model 6890N, Agilent technologies, USA) equipped with a cool on column (COC) injector and HP-1 column (see below). The replicates with similar GC profiles were combined and kept at -20 °C. Between collections, Porapak Q and Tenax TA tubes were rinsed with 4 ml diethyl ether and baked at least 2 h at 180 °C for Porapak Q and at 220 °C for Tenax, under nitrogen flow.

Volatiles retained in Tenax were directly analysed with thermal desorption on a GC with Optic inlet (OC) and HP-1 column (see below). Collections were used only once, for a tentative identification of compounds with GC-MS. The extracts from Porapak Q were used for gas chromatography-electroantennography (GC-EAG), gas chromatography-mass spectrometry (GC-MS) and gas chromatography- co injections.

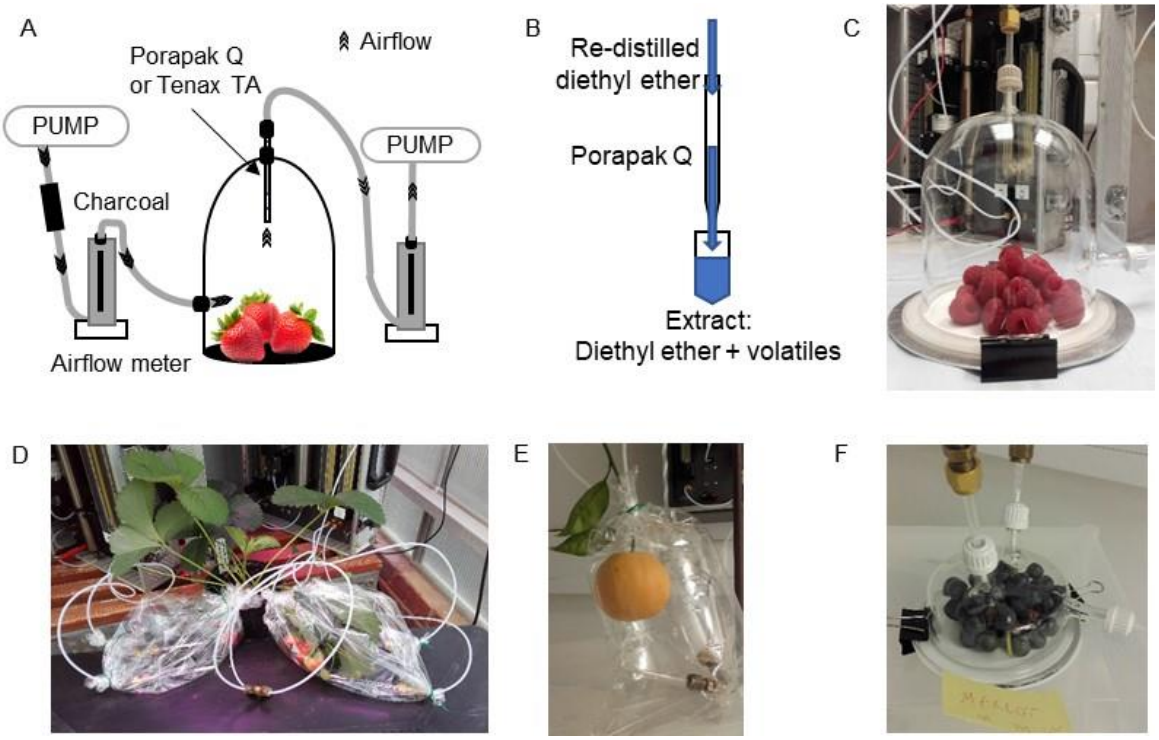


Figure 2-1 Dynamic headspace collection

A) Air entrainment system (DHC) to collect volatiles from harvested fruits in a glass vessel. B) Extraction of collected volatiles from the Porapak Q polymer. C) DHC of harvested raspberry fruits and grapes (F). Collection were done using roasting bags on strawberry plant (D) and a non-harvested orange (E) as examples.

2.5 ELECTROPHYSIOLOGICAL RECORDINGS

EAG responses are thought to be the summated depolarisation of all olfactory receptor neurons (ORNs) across the antenna sensitive to the tested olfactory stimuli. Bioactive compounds from the fruit headspace extracts were located with Gas Chromatography-Electroantennography (GC-EAG).This technique permitted to record the antennal activity of the insect to chromatographically separated peaks from the headspace extracts (Wadhams 1990).

Single sensillum recording (SSR) permitted to record extracellularly the activity of so-localised classes of ORNs in response to odorant stimuli (Kaissling 1995). This technique permitted was to identify which classes of ORNs were activated by fruit headspaces and by the identified fruit volatiles in gravid female *D. suzukii* (Figure 2-2).

2.5.1 Electroantennography (EAG)

A mated female was chilled in ice and the head excised. The antennal activity was measured by placing the recording electrode over the arista and an indifferent electrode at the base of the antenna within the head capsule. To obtain a reproducible preparation, it was necessary to record from the same location on each antenna. By cutting the tip of the arista off and placing the electrode over its remaining stump, the electrode contacted the olfactorily sensitive funiculus through the arista. Ag–AgCl glass electrodes were filled with saline solution (composition as in Maddrell *et al.* (1969) but without glucose). Signals were passed through a high input impedance amplifier (UN-06; Syntech, Hilversum, The Netherlands) and analysed using a customised software (Syntech). Responsiveness of the preparation was tested using a stimulus of 2-heptanone before and after the first recordings to ensure no responses were altered, particularly during GC-EAG runs (lasting 20 min).

GC-EAG was used to locate antennally active compounds from fruit extracts. Recordings were done with extracts collected (as above) from whole ripe fruit headspaces (see above) by Christine Woodcock (Rothamsted Research). The same technique was used by the author to obtain recordings with extracts from five grape cultivars (Chapter 5).

Separation of the volatiles was achieved on a GC (Agilent Technologies, 6890N) equipped with a COC injector and FID, using an HP-1 column (50 m, 0.32 mm ID, 0.52 µm film thickness). The oven temperature was maintained at 30 °C for 2 min and then ramped up at 15 °C /min to 250 °C. The carrier gas was helium. The effluents from the GC column were split and simultaneously directed to the antennal preparation and the GC FID detector. Outputs from the EAG amplifier and the FID were monitored simultaneously and analysed using the Syntech software package. The GC eluent was divided in a split, enabling its

simultaneous detection in the FID and the insect antenna. GC peaks which consistently elicited an electrophysiological response in two or more runs were considered active and annotated. Each EAG active peak could be characterized with a retention index from the GC allowing for further identification with GC-MS and co-injections. The EAG responses in this study were regarded as qualitative rather than quantitative hence, the amplitude of the depolarisation (mV) was not measured.

EAG responses were also detected within the time range of the eluting solvent, indicating that there are bioactive compounds masked by the solvent peak. These were tentatively identified from Tenax TA extracts using thermal desorption. Antennal responses from standards were measured with EAG in order to identify which of these compounds were bioactive.

2.5.2 Single Sensillum Recording (SSR)

The activity of ORNs from single basiconic sensilla on the antenna and maxillary palp of both fly species, was recorded extracellularly. A fly was immobilized in a truncated plastic pipette tip, with half of its head protruding from the narrow end and held on a glass microscope slide (Thermo Fisher Scientific, USA). The third antennal segment or maxillary palp were placed on a glass coverslip, steadily held with a glass micropipette. The preparation was kept under x1000 magnification (microscope Olympus BX51W1) in a charcoal filtered and humidified air stream (flux of 30 ml/s).

Action potentials generated by the ORNs present in each sensillum were recorded extracellularly, by inserting a glass electrode into the sensillum lymph, which surrounds the dendrites of the ORNs, and a glass reference electrode into the eye of the fly. Electrodes (<1 µm diameter) were filled with sensillum lymph ringer (Kaissling 1995) and placed over an AgCl-coated silver wire. The signal was led through a 10^{12} Ω input impedance amplifier (custom made) and were collected with InstruNet software (inet32dll version 3.3, Glenn Weinreb).

A 0.5 s air pulse (stimulus) was given through an odour cartridge into the air stream onto the fly. Signals were recorded for 10 s, starting 1 s before a stimulus was applied. The spontaneous activity (baseline) was recorded with an empty stimulus for each sensillum before testing odours. A delay was observed between the time the air pulse was triggered and the time the odour reached the antenna, corresponding to the travel time in the air stream. This delay was considered, starting the count of action potentials from the moment the odour reached the antenna. Upon reaching the antenna, the stimulus was diluted of

approximately 10-fold into the air stream. All recordings were performed on a minimum of three females per sensillum type. No more than 74 stimuli were applied to each fly.

The number of action potentials during a stimulus were counted for each neuron from the traces plotted with IGOR Pro (version 6.0.1.0, Wave Metrics Inc.).The neurons were identified in each sensillum denoting the neuron whose action potentials were the largest in amplitude by “A” and continuing to “D” for the neuron with the impulses of the smallest amplitude. Counts were standardized by subtracting the baseline activity for each neuron and were reported as a response rate in impulses/s.

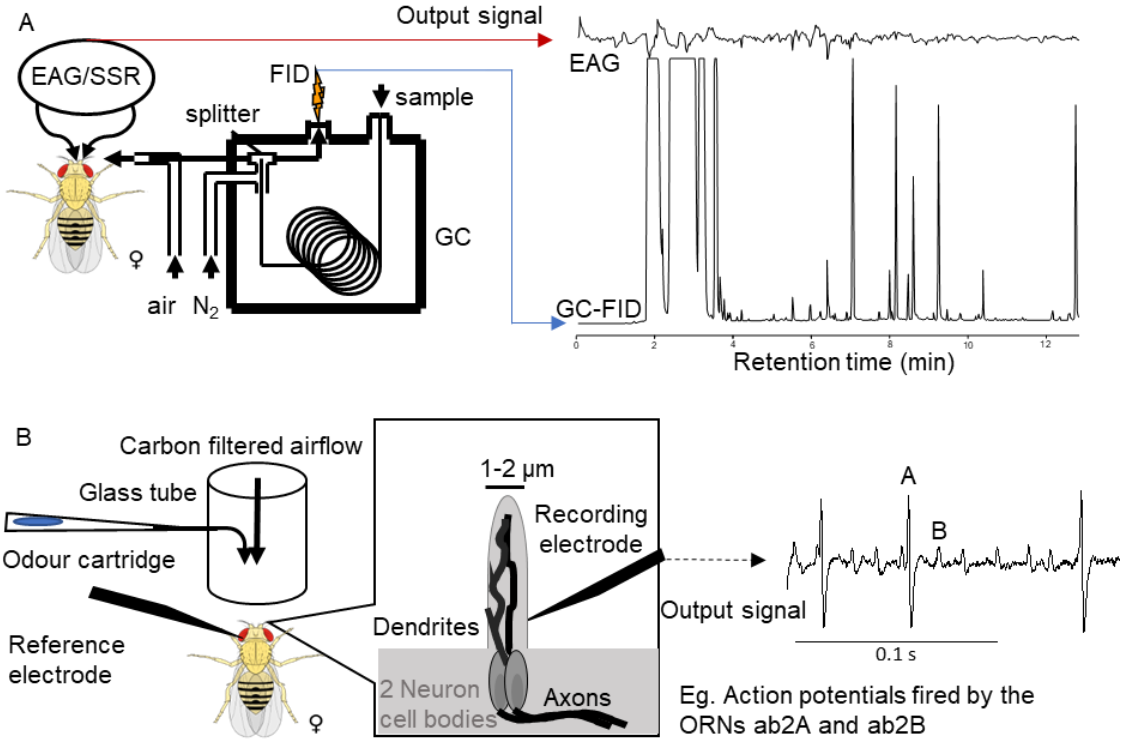


Figure 2-2 Electrophysiological recordings
A) Gas Chromatography- Electroantennography (GC-EAG) on *Drosophila flies*. B) Single Sensillum Recording (SSR) on a basiconic sensillum on *Drosophila* flies.

2.6 Gas Chromatography and Mass Spectrometry

Headspace volatiles collected from fruits were analysed using GC-EAG. Then, antennally active GC-FID peaks were then analysed with GC-MS to tentatively identify the active chemicals. The identity of headspace compounds was verified with co-injection, GC-EAG and EAG using synthetic chemicals.

ChemStation (version C.01.04 (35), Agilent Technologies, USA) was used for processing data from GC runs. Chemicals elute on GC column (below) with a specific time called the

retention time (RT). The elution speed of a compound depends on its interactions with the stationary phase of the GC column and can be characterised with a Kováts retention Index (KI). The KI can be calculated using the RT of the peak of interest and the RT of the two closest alkanes, ran on the same column (in the same condition as the sample is run) and referred to with known chemical libraries.

2.6.1 GC with HP-1 column

2.6.1.1 COC inlet

A GC with a cool on injector column (COC) allows to separate the compounds by their boiling point (see below). GC (model 6890N, Agilent technologies, USA) was equipped with a COC inlet, a Flame Ionisation Detector (FID) and a 50 m apolar capillary column HP-1 (50 m, 0.32 mm ID, 0.52 µm film thickness). The oven temperature was kept at 30 °C for 1 min, then increased by 5 °C /min until it reached 150 °C. After 0.1 min, temperature increased at a rate of 10 °C /min until it reached 250 °C and stayed constant for 20 min. Hydrogen was used as carrier gas. This GC equipped with a COC and HP-1 column was used for co-injection and quantification of compounds in the extracts, GC-EAG and GC-MS.

2.6.1.2 Optic inlet

Thermal desorption was used to extract chemicals from plant headspaces without using a solvent. It permitted to identify the most volatile compounds which were masked by the solvent required for other analyses. Chemicals absorbed on a Tenax TA polymer (see above) were desorbed using an optic inlet (OC) and were separated on a HP-1 column. This technique was used for a tentative identification of chemicals with GC-MS (see below).

2.6.2 GC with cyclodextrin or chiral column

To separate enantiomers, extracts were injected on a GC (Agilent Technologies, USA) equipped with a chiral β-cyclodextrin column (30 m, 0.25 mm ID, 0.25 µm film thickness), COC inlet and FID. The oven program was the following: temperature was constant at 30°C for 1 min then increased to 150 °C (5 °C /min) and held 0.1 min. Then temperature raised to 230 °C (10 °C /min) and held 22 min. The carrier gas was hydrogen. Each enantiomer from the racemic mixture eluted with a different retention time (RT), thus can be identified if present in the extract. In this study, (*S*)-Linalool and (*S*)- limonene were separated from their enantiomeric pairs (*R*)-Linalool and (*R*)- limonene.

2.6.3 Gas Chromatography- Mass Spectrometry (GC-MS)

The tentative identification of chemicals from bioactive GC-FID peaks located with GC-EAG, was performed by John Caufield (Rothamsted Research) with GC-MS. Extracts from porapak Q collections were run on a GC (HP 5890 series II) coupled with a Mass Selective detector or mass spectrometer (MS, HP). The GC was equipped with a COC and HP-1 column (50 m, 0.32 mm ID, 0.52 µm film thickness). The temperature was held at 40°C for 1 min then raised to 250°C (5°C /min) and held for 17.2 min. Samples from Tenax TA were analysed by thermal desorption with a GC with OC inlet and HP-1 column. The temperature was programmed to start at 30 ^{0}C then rise to 250 ^{0}C at 16 ^{0}C /s.

GC were coupled with a Micromass Autospec Ultima, magnetic sector mass spectrometer, equipped with a PTV unit (ATAS GL) and Agilent 6890N GC (fitted with a non-polar HP1 column 50 m length x 0.32 mm inner dia. x 0.52 µm film thickness, J & W Scientific). Ionization was by electron impact (70 eV, 220 ^{0}C). The GC oven temperature was maintained at 30 ^{0}C for 5 min and then programmed at 5 ^{0}C /min to 250 ^{0}C.

2.6.4 Co-injection

Co-injections were performed for the verification of the identity of components from the extracts. Synthetic standards of tentatively identified compounds were combined into a blend in similar quantities found in extracts. The identity of the compounds was validated when the synthetic standard gave a similar GC performance (KI) and EAG response as compounds in the extracts. Then, synthetic standards were co-injected with the extract on a GC. Peaks of the compounds of interest from the extract and the co-injected extract with blend must align: i) their KI and their width should be similar and ii) their height should have doubled. Synthetic standards which were successfully co-injected and elicited an EAG response were confirmed as fruit headspace volatiles, detected by mated female.

2.7 BEHAVIOURAL ASSAYS

2.7.1 Oviposition assay

Fruit host status for egg laying was determined with a no-choice oviposition assay. Females and males were housed together for 24 h in clear 28.5 x 97 mm polystyrene vials (Dutscher SAS, France) containing moist cotton, opened on the surface of undamaged fruit skin (approximately 0.78 cm^2). Fruits were considered hosts if the females laid eggs in them (Figure 2-3).

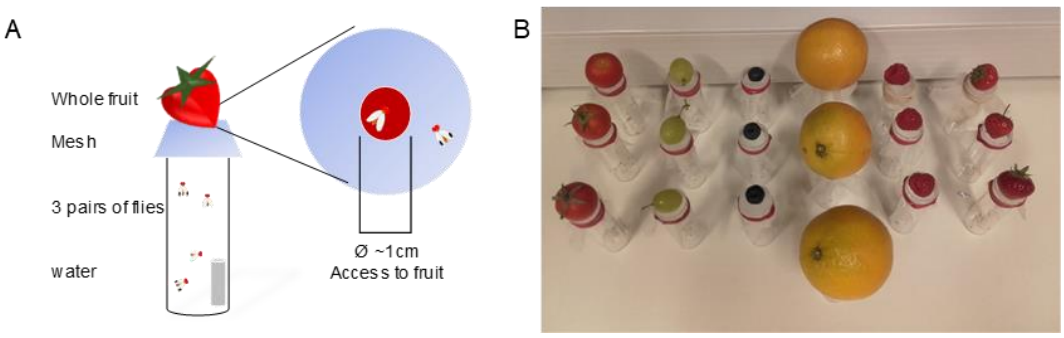


Figure 2-3 Oviposition assay with whole ripe fruits

A. Scheme of oviposition assay on whole fruits. B. Oviposition assay with six different fruits (left to right): Tomato, grape, blueberry, orange, raspberry and strawberry.

2.7.2 Olfactory behavioural assays

Two olfactory bioassays were designed to test the behavioural response to fruit headspaces by gravid females *D. suzukii*. The two presented below were of the highest efficacy with a behavioural decision (choice) scored for more than 80 % of the females in most cases (Appendix 1). Females were deprived from oviposition and food substrate for 8-12 h (supplied with moistened cotton wool only) prior to testing to stimulate their foraging behaviour. The deprivation stimulated the female response in the assay, increasing the response rate compared to not deprived females (Appendix 1).

2.7.2.1 The 4-choice cage assay

A 4-choices assay was used to assess the behavioural response of *D. suzukii* females to the odour of fruits and synthetic chemicals in a short-range assay with no induced directional airflow (Figure 2-4). Four beakers containing a same amount of most cotton wool, were placed in each corner of an insect rearing cage (W 30 x D 30 x H 30 cm) covered with aperture mesh (1.35 µm) (BugDorm-1, MegaView Science Co., Ltd). One of the beakers (the treatment) contained fruits or chemicals, the three others were the control (containing water or solvent). The content of each beaker covered with a piece of white paper and was not visible from the top. Beakers were closed with a mesh allowing odorants to diffuse through and preventing flies from entering. A custom-made aluminium platform created a floor at the same level as the top of the beakers. The platform was perforated with 5.7 cm diameter holes on each corner on top of each beaker revealing the mesh. On top of each beaker an arrestment bait made of a cotton roll soaked with 10 % sucrose solution. It permitted to retain females on top of the beakers where they were collected (Appendix 1).

Five replicates (one cage per replicate) ran at the same time. Cages were aligned on a table in a lighted room. 10 replications were done for each experiment with 10-15 deprived gravid females. Flies on top of the baits were collected from the apparatus each hour during 5 h

and scored. Flies on controls 1, 2 and 3 were scored clockwise from the treatment bait position. A randomisation of the bait placement removed a possible orientation bias. Females which remained in the cage (above and below the platform) at the end of the experiment were scored as “No-choice”. Experiments lasted 5 h unless otherwise indicated.

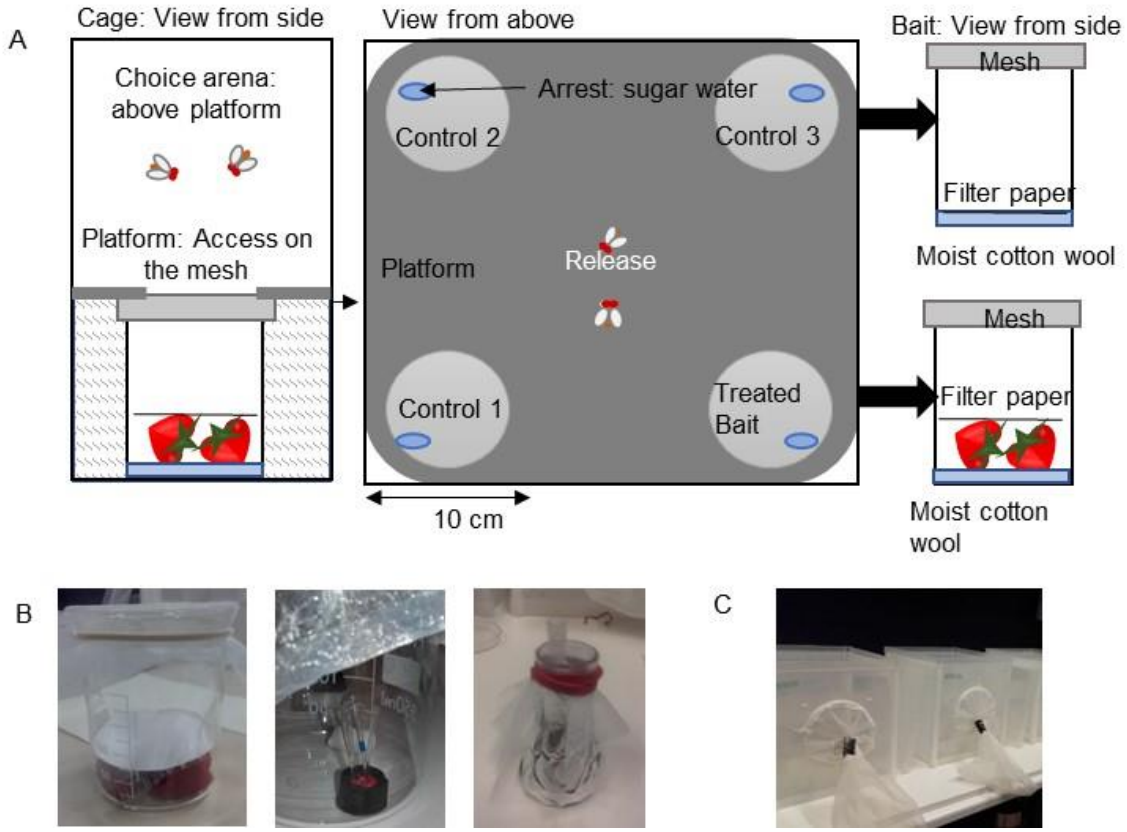


Figure 2-4 The 4-choice cage assay

A) The 4-choice cage assay experimental setup B) Baits: strawberry fruits (left) and chemicals in glass capillaries (right). C) Cages aligned next to each other during the experimental trial.

2.7.2.2 Multiple choice assays

In a first experiment, the preference between three different fruit types and one control was assessed by presenting three fruits: raspberry, grape and blueberry, and one control simultaneously in a cage assay as above. In a second experiment visual stimuli were included: the same fruits were presented in Petri dishes positioned on each corner of a rearing cage (as above). Fruits were visible and accessible to the flies. Both experiments were replicated 10 times with 10-20 deprived gravid females and lasted 5 h.

A third multiple choices assay was used to test preferences among five grape cultivars. Grapes (with stem still attached to reduce the mechanical damage on the fruit) were kept in

50 ml Erlenmeyer flasks wrapped with aluminium foil to conceal the content. Flasks were closed with a mesh allowing the volatiles to diffuse through and punctured with a pipet tip serving as a trap entrance. Five flasks containing each one of the cultivars and one flask containing only water were evenly positioned on the floor of a rearing cage (as above). The position of the flasks was randomised. Eight replications were done with 10-20 deprived females during 24 h. Flies found outside the flasks at the end of the experiment were counted as “No choice”.

2.7.2.3 Wind tunnel

An evenly lit wind tunnel (W 90 x D 30 x H 30 cm) with a directed 0.25-0.35 m/s airflow was used to assess the attraction of female *D. suzukii* toward fruits and chemicals over longer distance (90 cm from the odour source) compared to the 4-choice cage assay (above, 15 cm from each odour source). The floor had a black and white striped pattern to provide visual stimuli for the flying insects. Flies were released on a platform approximately 15 cm up on the downwind side. On the upwind side, two platforms serving as a treatment bait and as a control bait were placed next to each other (approximately 5 cm apart) at a height of approximately 10 cm. The treatment held fruits or a chemical dispenser (see above) and the control held water or a solvent. Baits were constructed as follow: odour sources were positioned on Petri dishes (90 mm diameter) covers, filled with moist cotton and a white filter paper (Whatman, type I). Petri dishes were surrounded with a piece of white paper (5 cm wide) preventing females from seeing the fruit from their starting position. However, in-flight females could see the fruits so visual cues were not totally discarded from the experiment. Bait positions were swapped around between replications to remove any side bias. N= 4-8 replications with 12-50 mated females were done during 5 h (Appendix 1). Females which landed on platforms were collected each hour from the apparatus and scored. The walls and floor of the wind tunnel was cleaned with water and ethanol and baits replaced between replications.

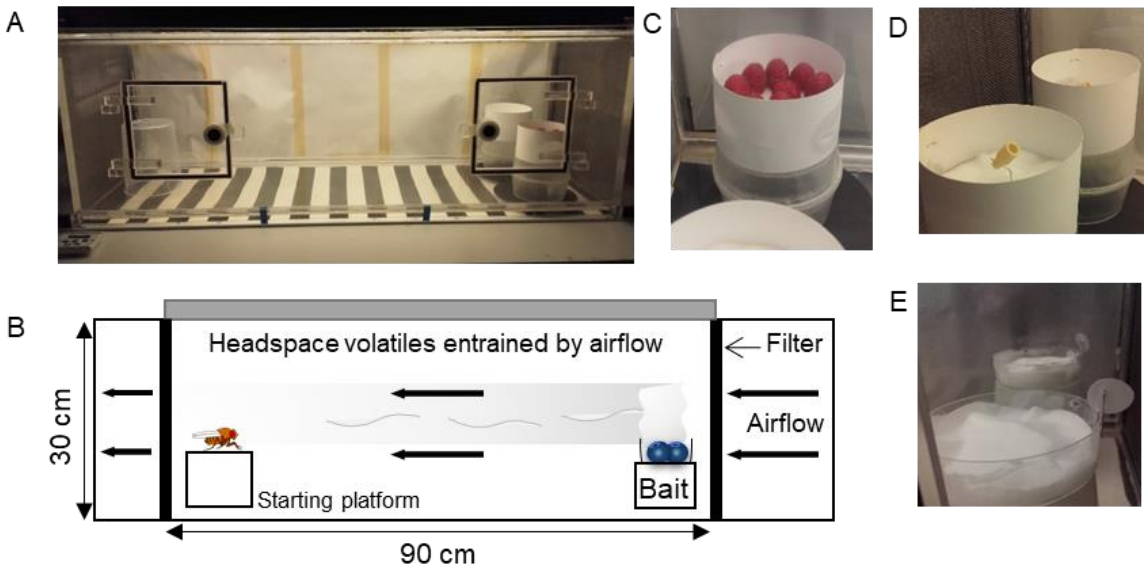


Figure 2-5. Wind tunnel assay to test D. suzukii attraction to fruit headspace.
A) Picture of the wind tunnel. B) Scheme representing the major parts of the bioassay. C-E) Baits made of whole fruits (C) and dispensers such as rubber septa (D) and filter paper (E) to test chemicals.

2.8 DATA ANALYSIS AND GRAPHICAL REPRESENTATION

Statistical analysis and graphics were done using Graph Pad Prism (version 8.1.1, Graph Pad Software Inc. USA), R 3.2.0 (The R Foundation for Statistical Computing) and SPSS (version 25, IBM Corp., USA). Graphics and schematics were done with Microsoft Office 365 (version ProPlus, Microsoft, USA) and ACD/ChemSketch (version 2018.1, Advanced Chemistry Development, Inc., Canada). Guidance for statistical analysis and use of R were kindly given by S. Powers (Stats Powers, UK) and K. M. Schmidt (School of Mathematics, Cardiff University).

2.8.1 Statistical analysis on electrophysiological data

For each class of ORNs, the response rates to fruit headspaces was compared to the response rates to control. The distribution of values for each variable (fruit headspace and ORN class) was tested for normality, using the Shapiro-Wilk normality test and a Normal QQ plot was plotted beforehand to select the statistical model (Appendix 2 and 8).

The activation of classes of ORNs when exposed to fruit headspaces and control were compared within species using a repeated pairwise Wilcoxon signed-ranked test. The activation between species (Chapter 6) and populations (Chapter 7) were compared using Mann-Whitney U-test (also called Wilcoxon sum-ranked test) followed by Holm-Sidak correction for multiple comparisons. The intensity of responses between cultivars were

compared using a Kruskall-Wallis multivariate analysis and, in case of significance were followed by Mann-Whitney post-hoc pairwise comparison, adjusted with Bonferroni multiple comparison correction.

The difference in response between ORNs from two populations of flies (Chapter 7) were graphically represented using the following formula:

$$D = \frac{1}{n}\sum_{i=1}^{n} Hi - \frac{1}{n}\sum_{i=1}^{n} Li$$

With H= (H1, ..., Hi) and L= (L1, …, Li) the responses to the same stimulus by the two homologous ORNs from populations H and L.

2.8.1.1 Hierarchical Cluster Analysis

Responses of ORNs were classified using an agglomerative hierarchical cluster analysis (HCA). Average responses from ORNs were standardised as Z-scores (from -1 to 1) then, were used as multidimensional coordinates to compute the Euclidian distance between pairs of ORNs. Dendrograms were created using Ward's method on squared Euclidian distances. Differences between overall ORN responses were graphically represented as the Euclidian distance. The Euclidian distance was calculated from the Pythagorean formula over multiple orthogonal dimensions:

$$d(a,b) = \left| \sqrt{\sum_{i=1}^{n} (bi - ai)^2} \right|$$

With a = (a1, ..., ai) and b= (b1, …, bi) the two Cartesian points in a Euclidian n- dimensional space.

2.8.1.2 Dose response relationships

Dose response curves were fitted with a 4-parameter Hill curve as follow:

$$Y = R_{min} + (R_{max} - R_{min}) / (1 + (10\text{^}(LogD_{EC50} - X)) * n_H)$$

With R the response rate (impulses/s), D_{EC50} the dose which gives half the maximal response (R_{max}), and n_H the Hill coefficient (or slope). X is expressed as $\log_{10}$ (dose).

2.8.2 Quantification of chemical components from GC extracts

From extracts an estimation of the magnitude of quantities of chemicals released by fruits could be made. A solution of alkanes (C6-C20) of known concentrations was run on the same column (and program) as the extract. Using the height of the peak of the

compound of interest, relative to the height of the peaks from the two closest alkanes (which corresponding quantity is known), the quantity of chemical was determined. As the extract had been previously concentrated (see above), chemicals may have evaporated along with the solvent and therefore it is not possible to have an accurate measurement of how much chemical was released by the fruit. The quantities reported are an estimate which indicate the magnitude (e.g. 10, 100 or 1000 mg) rather than exact amounts.

2.8.3 Statistical analysis on behavioural data

Behavioural data were counts of females collected on various baits, controls and no-choice (remaining in the cage at the end of the experiment). Their distribution followed a Poisson distribution, hence were analysed with non-parametric models. Preference indices (see below) were standardised measures hence could be analysed using parametric tests.

2.8.3.1 The 4- choices cage assay

The total number of females collected on four spots: one bait, three controls, and in the cage (no choice) were summed at the each of each trial. Data were represented as numbers of females collected on the bait, control and no choice in graphs. Firstly, the number of females collected on the three controls were statistically compared (see below) and were average if not significantly different (Appendices 4 & 6). Then, the numbers of females on bait, control and no-choice were statistically compared.

Data were analysed in two steps using a General Linear Model (GLM) with Poisson distribution: i) the number of females on the three controls were compared to test the hypothesis stating that the three controls were similar enough to be randomly chosen by females. If true, the three controls were pooled together then; ii) the number of females on bait, pooled controls and no-choice were compared to test the hypothesis that females randomly distributed in the cage (on bait, control or no-choice). Various factors related to the experimental conditions, included the time course of the test, abiotic conditions, spatial orientation of baits, fruit cultivars, were randomized in the experimental procedure. They were included in a first model before to be removed as they did not influence the experimental outcome.

A GLM was fitted following Nelder and Wedderburn (1972):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + ... + \beta_n X_n + \varepsilon_i$$

where Y is the response to be modeled, X_1 ... X_n are the model variables with β_1 .... β_n the corresponding regression variable to be fitted. β_0 the intercept, and εi the random residuals

of the Y (i runs through the observed instances). A linking function is then applied to fit the distribution of the data (Poisson distribution) with the responses of the linear model.

The model variables include the behavioural responses (i) or (ii) of the female and the position of the bait, the total number of females used per replication, biotic (age of females, deprivation state) and abiotic (temperature, humidity) conditions of the experiments.

The model is fitted under the assumption that the mean E(Y) is equal to the variance var(Y) of the response Y. Sometimes the model is not appropriately fitted, likely because relevant explanatory variables may be omitted or because of a large proportion of zeros in the data. This causes overdispersion, which is a deviation of the mean compared to the variance. Where necessary, overdispersion was resolved by correcting the deviation using a quasi-likelihood estimation (or quasi-Poisson).

2.8.3.2 Multiple-choices assays

In Multiple choice assays the numbers of females collected on each bait were compared using a GLM with Poisson distribution (as above). Numbers of females were compared between each bait and control for each experiment. Where applicable, the model also included the effect of exposure and its interaction with the choices offered (Appendix 4).

2.8.3.3 Wind tunnel

The total numbers of females collected during the wind tunnel assay, downwind, upwind on the two platforms, were recorded as binomial factors for each replication as follow: i. the number of females reaching the platforms (N upwind), and the number of females not responding (N downwind); ii. females upwind were scored as the number of females choosing the fruit or odour (N bait), and the number of females choosing the control (N control). The distributions of females were compared using a binomial GLM following the above procedure.

Responses were graphically represented as a preference index (PI):

$$\text{(i)} \quad PI = (N_{upwind} - N_{downwind}) / (N_{upwind} + N_{downwind})$$

$$\text{(ii)} \quad PI = (N_{bait} - N_{control}) / (N_{bait} + N_{control})$$

PI were statistically compared to a null Hypothesis H_0, using a Sample t- test. H_0: PI=0, there was no preference for either bait or control.

2.9 IMAGING

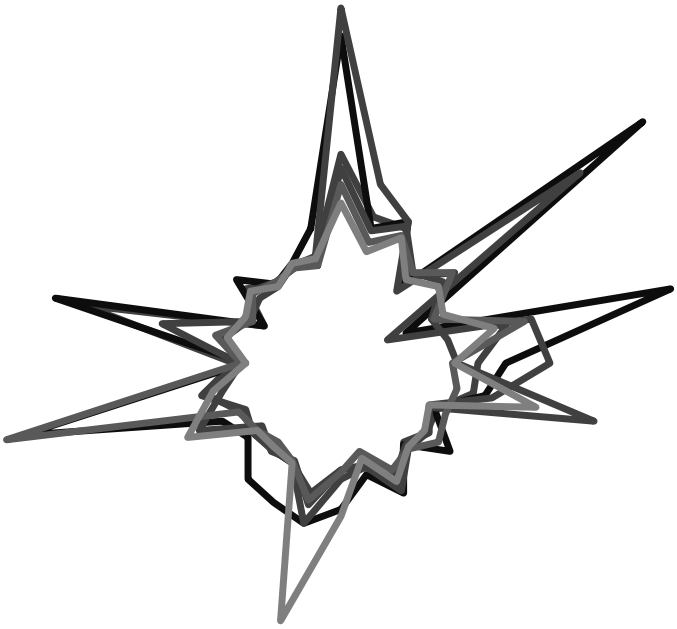
Scanning Electron Microscopy (SEM) of the peripheral olfactory system was performed by Rebecca Lauder (Rothamsted Research, UK). Adult females were kept at -20 ºC for 30 min

prior to imaging. They were attached to aluminium stubs using conductive carbon adhesive discs (TAAB) and a 50:50 mixture of TissueTek: colloidal graphite. Samples were plunge frozen in liquid nitrogen and transferred to the GATAN ALTO 2100 cryo prep system. They were etched and coated in a thin layer of gold. Micrographs were collected using a JEOL JSM 6360 scanning electron microscope at 5kV under cryo conditions.

Pictures of adult flies on fruits were taken in laboratory conditions by Graham Shephard (Rothamsted Research). All other pictures, graphics and schematics were originally created by the author, Claire Duménil, unless stated otherwise. Adults anatomy, eggs and larva in fruits were taken using a Leica M205 stereo microscope (Leica Microsystems, UK).

CHAPTER 3

3 FRUITPRINTS: ENCODING OF FRUIT ODOURS BY THE PERIPHERAL OLFACTORY SYSTEM IN *DROSOPHILA SUZUKII*



"Fruitprints" or abstract representation of the activation of neurons by six fruit odours on the peripheral olfactory system of *Drosophila suzukii*.

3 FRUITPRINTS: ENCODING OF FRUIT ODOURS BY THE PERIPHERAL OLFACTORY SYSTEM OF *DROSOPHILA SUZUKII*

3.1 INTRODUCTION

3.1.1 Aim of Chapter

Using *D. suzukii* as a neuro-ethological model, the aim of the research presented in this chapter is to understand how host cues are encoded by the olfactory receptor neurons (ORNs) in a species that uses a variety of plant hosts for oviposition. Based on current understanding of odour coding by the olfactory system, the following hypotheses are addressed: i) host recognition involves the activation of ORN combinations, which are specific to fruits from each plant species; ii) hosts are recognised by the activation of a common subset of ORNs, which differentiate them from non-hosts (Figure 1-6).

Guided by previous research on *D. melanogaster*, 28 functional classes of ORNs were identified on the antennae and maxillary palps of female *D. suzukii*. Their responses to ripening fruit odours were characterised using a selection of fruits from six taxonomically distant plant species, whose host status was assessed with olfactory and oviposition assays. Harvested whole ripe fruits were used in order to obtain volatile profiles corresponding to fruits which had undamaged skin, as it would be the case when ripening on the plant (Introduction, 1.1.2). The assays were specifically developed to test *D. suzukii* behavioural response to fruit odours (Appendix 1). Preference among fruits from different plant species and their cultivars were described.

The study suggests that a subset of seven ORNs are dedicated to oviposition site selection. Combinatorial patterns of activation, so called fruitprints enable females to discriminate among fruit substrates for oviposition. Four of these were commonly activated by at least two of the host fruits hereby showing a host versus non-host recognition. The study also suggests that an innate preference-ranking strategy enables *D. suzukii* to select suitable oviposition substrates throughout the year and is associated with visual cues.

3.1.2 Background

The peripheral olfactory system was described in *D. melanogaster* and homologous structures were named similarly in other Drosophilids. Olfactory receptor neurons (ORNs) that are housed in peg-like or hair-like structures on the surface of the antenna and maxillary palps. Functional structures called basiconic sensilla house two to four ORNs tuned to food/host odours (Introduction, 1.3). ORNs are associated with olfactory receptors (ORs) on their dendrites which are tuned to few or many different food and host volatiles.

Approximately 13 functional types of basiconic sensilla have been identified, labelled ab1, …, ab10 on the third antennal segment and pb1, …, pb3, on the maxillary palp. The ORNs can be recognise by the amplitude of their action potentials on electrographs and are labelled according to this amplitude and to the housing sensillum type (Method, 2.8.2). For instance, the basiconic sensillum type ab1 house four ORNs: ab1A, ab1B, ab1C and ab1D. These ORNs once activated, relay sensory signals towards centralised olfactory units (Introduction, 1.3).

Classes of ORNs detects odorant molecules with different affinity as some ORs are broadly tuned while others are narrowly tuned, even at high doses (Hallem and Carlson 2004). Concentrations and ratios of odorant molecules within an odorant plume also modulates how ORNs detect host odours. The resulting information from their encoding is transported to the antennal lobe in which information is modulated by interaction between glomeruli (Introduction, 1.3). This way, the olfactory system has evolved the possibility to encode complex chemical signals, using a limited number of sensory units. The encoding of olfactory information on the ORNs and their processing within glomeruli can be measured and quantified and is well described in *D. melanogaster (Introduction 1.3)*.

3.1.2.1 Fruit detection in D. melanogaster

The affinity of ORN classes in *D. melanogaster*, was described with hundreds of natural occurring odorants, summarised on DoOR (Münch and Galizia 2016). To date, 34 fruit scents have been tested on the repertoire of 48 ORs of the peripheral olfactory system of *D. melanogaster* (Dweck *et al.* 2018). This study showed that over 50 % of all classes of ORNs were activated by fruit volatiles. In addition, food odour specificity of ORNs on maxillary palps was shown with the detection of several fruit and faeces scents. Over 500 single odours have been tested with SSR and induced a response of at least one of the OR, hereby testing over 10,000 ligand-OR combinations (Kreher *et al.* 2005; Kreher *et al.* 2008; Mathew *et al.* 2013; Dweck *et al.* 2018). These studies enabled to hypothesise how the olfactory system encode diverse odours which lead to specific behaviours. The responses of ORs to individual components were also linked to behavioural attraction to fruits in *D. melanogaster*, suggesting that each ORN class is dedicated to specific ecological relevant roles. For instance, attractive behaviour and host specialization in three *Drosophila sp.* were linked to the activation of ab3A (Dekker *et al.* 2006; Linz *et al.* 2013; Mansourian *et al.* 2018).The attraction to citrus fruits was shown to be mediated via the detection of terpenes by the AI2A (Dweck *et al.* 2013). Other pathways were highlighted, for instance, cues associated with danger, inducing a repellent behaviour were mediated via specialised olfactory circuitry: The ab4B detects the chemical geosmin released by harmful bacteria

(Stensmyr *et al.* 2012); The ab10B detects *Leptopilina sp*. parasitoid wasp body odour components: nepetalactol, (*R*)-actinidine and (*R*)-iridomyrmecin (Ebrahim *et al.* 2015).

3.1.2.2 Fruit detection in D. suzukii

Similarities with *D. melanogaster* guide the studies on *D. suzukii* but their different ecological niches likely implies the use of different olfactory cues. For example, these can be cues that are associated with ripening fruits attached in the foliage of the plant instead of fermenting cues associated with the decay of overripe fruits on the ground (Keesey *et al.* 2015). Therefore, a species-specific characterisation of olfactory units dedicated to host selection in *D. suzukii* would be valuable to develop species-specific management tools. Because these species are closely related, odours and mechanisms that have been found relevant to the former, are interesting starting points to start the study host selection in *D. suzukii*.

The ORNs housed in basiconic sensillum types (ab1-ab10 and pb1-pb3), which were identified as the main host/food detecting units in *D. melanogaster*, remain to be fully described in *D. suzukii*. The genes coding for the olfactory receptors (ORs) that are expressed in these functional sensillum types were recognised in *D. suzukii* and most of them were conserved (Hickner *et al.* 2016; Ramasamy *et al.* 2016).

Behavioural attraction via olfactory cues has also been studied to some extent. Indeed, several electrophysiological and behavioural studies showed the role of olfaction in host fruit detection, via recordings of the whole antennal activity (with electroantennograms, EAGs) when ripe fruit odours were presented (Abraham *et al.* 2015; Revadi *et al.* 2015). Various volatiles of aldehydes alcohol and esters induced antenna activity and showed little overlap between different host fruit types tested (raspberry, strawberry, cherry and blackberry) even though most of them were from the Rosaceae family, hence taxonomically close. In addition, single sensillum recordings (SSR) on the functional basiconic types ab1, ab2 and ab3 revealed that *D. suzukii* is more sensitive than *D. melanogaster* towards several ripe fruit odours, including methyl butanoate, methyl isopentanoate, butyl acetate, isopentyl acetate, and hexyl acetate (Keesey *et al.* 2015). Several other odorant volatiles were identified as behaviourally relevant to the flies including odours originating from fermenting activity such as acetic acid, ethanol (Cloonan *et al.* 2018).

The sensitivity of *D. suzukii* to both ripening and overripe fruit odours confer an interesting position to the fly, with two distinct ecological nice in which they can survive. They are indeed attracted to volatiles that are specific to both host types, indicating that the evolutionary shift may be ongoing. Yet, the preference for ripening fruits is clear (Keesey *et al.* 2015; Cloonan *et al.* 2018).

In addition, the functional type ab3 was suggested to mediate host specialisation in *D. suzukii*. It was found to mediate attraction to the volatile β-cyclocitral in field trap assays and its tuning to fruit volatiles had adapted, with increased sensitivity for ripening fruit odours (Schlyter *et al.* 2012; Keesey *et al.* 2015; Ramasamy *et al.* 2016). These shed light on a positive correlation between olfactory detection of green leaf associated volatiles, and a behavioural adaptation to ripening fruits in the tree canopy. This is the fifth *Drosophila spp.* for which the ab3A appeared tuned to host specific cues. This ORN class was associated with host shifts in *D. melanogaster* (Mansourian *et al.* 2018), *D. orena* (Comeault *et al.* 2017), *D. sechellia* (Stensmyr *et al.* 2003; Dekker *et al.* 2006) and *D. erecta* (Stensmyr *et al.* 2003).

3.2 THIRTEEN FUNCTIONAL BASICONIC SENSILLUM TYPES HOUSE 28 CLASSES OF ORNS RESPONDING TO FOOD/HOST ODOURS

Following comparative studies, it was hypothesized that homologous of the classes of ORNs housed in the functional types of basiconic sensilla could be identified in *D. suzukii*, using similar techniques, and diagnostic panels of chemicals as used in prior studies with *D. melanogaster*. Sensillum types were characterised by the response of their associated ORNs to a panel of odorants during single sensillum recordings (SSR). Diagnostic odour panels allowed the recognition of corresponding basiconic sensillum types in *D. suzukii*. To indicate this functional correspondence, the sensilla were named ab1-ab8 and ab10 as in *D. melanogaster*. In *D. suzukii* a sensillum corresponding to ab9 was missing, but an additional sensillum type was found and was called abX. In the maxillary palps, three sensilla types were found, which correspond to the three main types in *D. melanogaster*. These were characterised by W. van der Goes van Naters using a different panel of ligands. As for *D. melanogaster*, the ab1 housed four ORNs (ab1A, ab1B, ..., ab1D) while all others housed two ORNs (e.g. ab2A and ab2B).

3.2.1 Characterisation of 28 classes of ORNs dedicated to food odours

Key chemicals allowed immediate functional identification of a sensillum type because one of the ORNs was particularly sensitive to it and this ligand was not equally active on other sensilla (Figure 3-1). These key ligands are CO_2 for ab1C, ethyl acetate for ab2A, 2-heptanone for ab3B, (*E*)-2-hexenal for ab4A, pentyl acetate for ab5B, (*RS*)-1-octen-3-ol for ab6A, ethyl lactate for ab7A, ethyl butanoate for ab8A, 2-phenylethanol for ab10A, and (*RS*)-linalool for abX. Responses to these key ligands were measured across all sensillum types as were responses to 16 other chemicals, chosen for their activity on ORNs in *D. melanogaster* (Appendix 9).

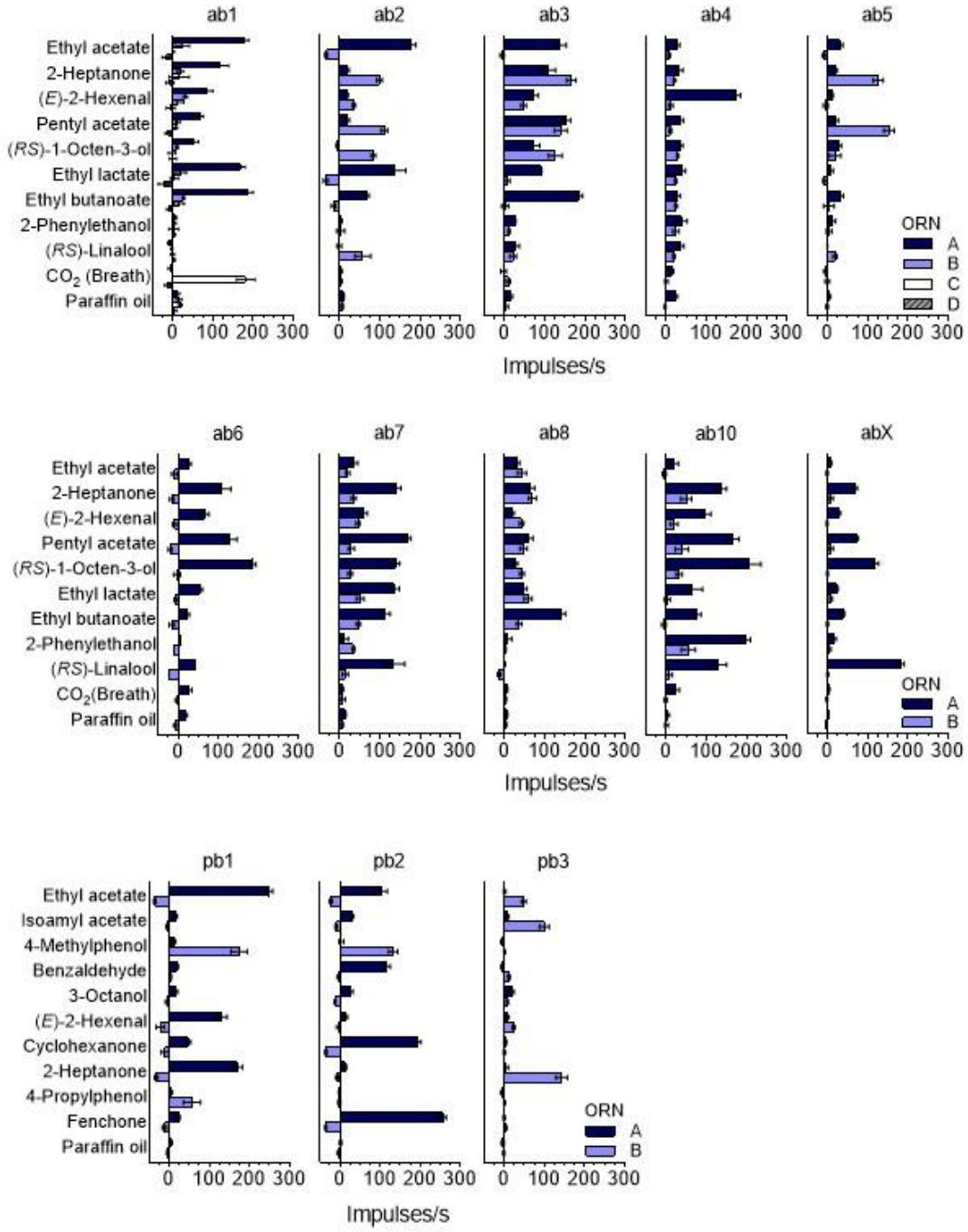


Figure 3-1 Characterisation of antenna and palp basiconic olfactory receptor neurons (ORNs) in D. suzukii.

Sensillum type on the antenna: ab1,…, abX and on the maxillary palp: pb1, pb2 and pb3. ORNs are labelled A, B, C or D by the decreasing amplitude of their fired action potentials (impulses). Mean ± SEM impulse rate during a 0.5 s stimulus. Stimuli consisted of an air pulse through a glass cartridge containing 30 µl of a 1% v/v dilution in paraffin oil. Unless stated otherwise, chemicals were racemic. The CO_2 stimulus was a glass cartridge filled with exhaled air. The spontaneous activity was subtracted from all responses. N= 8-15 sensilla for each functional type; recordings were made from at least three females for each functional type.

3.2.2 Position and numbers of basiconic sensilla on the antenna

Basiconic sensilla in the antennae can be divided into two size groups: the large basiconic sensilla (ab1-ab3), that are concentrated medially, while the small basiconic sensilla (ab4-ab8, ab10 and abX) occupy more lateral and distal regions of the antenna where they intersperse with trichoid and coeloconic sensilla. The regionalisation of large and small basiconic sensilla was observed under a scanning electron microscope (Figure 3-2).

Under the light microscope, functional types can be differentiated by their shape and size: the ab1 tip is wider than of neighbouring types, ab3 are the thinnest among neighbouring ab2 and ab1. The type ab4 appear to be the longest among small basiconic sensilla. The type ab7 can be recognized among the small basiconic sensilla by a wider shape, whereas, the type abX appears to be the thinnest and shortest.

The functional types are arranged in partially overlapping, regions on the funiculus (Figure 3-2). The large basiconic types are only distributed in the medial anterior and posterior region. The ab1 are located with a higher density on the distal side of the “large basiconic area”. The ab4 are mostly found near the distal side of the “large basiconic area”, sometimes overlapping with the ab1. The ab8 are mainly found in the most proximal region of the antennal segment. The types ab5 and ab10 are almost always found near the distal tip of the funiculus. Other small basiconic sensilla are found at the distal tip, most proximal basal region on medial, anterior, posterior and lateral regions, mostly mixed.

The functional types are found in different proportions: large basiconic sensilla represent 60 % of all antennal basiconic sensilla, with the most abundant being the ab2 (30 %), followed by ab1 and ab3. Ab7 and ab8 each represents about 10 % of the antenna population. The type ab9 was not identified in *D. suzukii*.

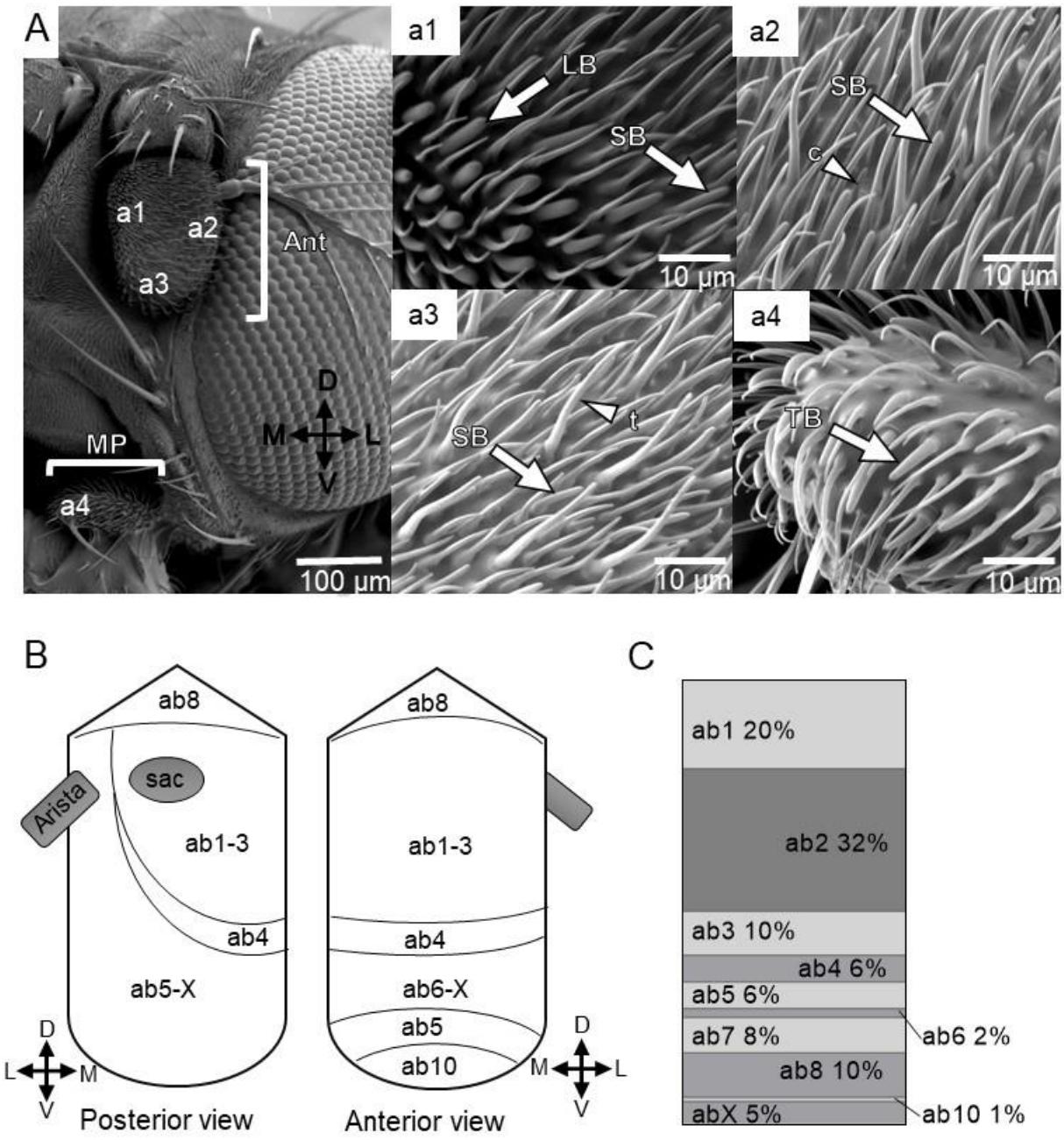


Figure 3-2 Mapping of functional basiconic sensillum types in D. suzukii

A) Scanning Electron Microscopy of the frontal view of the head exposing the frontal side of the third antennal segment or funiculus (Ant) and the lateral side of the maxillary palp (MP). Large (LB) and small (SB) basiconic sensilla on the antenna are indicated in panels a1 (dorsomedial side), a2 (dorsal side), a3 (ventral tip). The thin basiconic sensilla (TB) on the maxillary palps are shown in panel a4. Trichoids ("t") and coeloconics ("c") are visible among the small basiconic sensilla on the antenna. Orientation arrows: "D" dorsal, "M" medial, "L" lateral and "V" ventral. Pictures are a courtesy of Rebecca Lauder, Rothamsted Research. B) Anterior and posterior schematic views of the third antennal segment with the regionalised position of the functional basiconic types identified with SSR under light microscopy. Basiconic types were identified by the responses to odour panels from their housed ORNs. "sac" Approximate location of the sacculus. Lines

schematically delimit the areas where the types predominate. C) Proportions of each functional basiconic types on the whole funiculus on a total of 189 sensilla recorded.

3.3 SEVEN FRUIT-ACTIVATED CLASSES OF ORNS IN D. SUZUKII

The responses to six host and non-host ripe fruit odours by the 28 classes of ORNs characterised above were measured on gravid females *D. suzukii*. Fruits gave characteristic activity patterns across the ORN classes which were called “fruitprints” (Figure 3-3). Three hosts are strawberry, raspberry (Fam. Rosaceae) and blueberry (Fam. Ericaceae). Grapes (Fam. Vitaceae) are accepted as host depending on the cultivar and non-hosts are orange (Fam. Rutaceae) and tomato (Fam. Solanaceae) (CABI 2018; EPPO 2018). Their attractiveness and susceptibility to egg laying was also assessed.

3.3.1 Fruitprints on the peripheral olfactory system of *D. suzukii*

The fruitprints can be described by the seven ORNs with the highest activations, some of which are fruit specific (Figure 3-3). The following activities correspond to an increase compared to the spontaneous activity. Strawberry headspaces activated pb1A, ab1A, ab2A, ab3A and abXA, with more than 100 impulses/s. Raspberry activated ab1A and ab2A with more than 100 impulses/s and ab3A with 36-45 impulses/s. Blueberry headspaces activated pb1A with 67-82 impulses/s and ab1A with 39-60 impulses/s. Grape headspace activated ab4A with 64-82 impulses/s and ab1A with 39-45 impulses/s. Orange headspaces activated abXA with more than 100 impulses/s and ab3A 35-53 impulses/s. Lastly, tomato headspaces activated ab7A with 68-84 impulses/s and ab4A with 34-56 impulses/s. Other ORN response rates were all below 40 impulses/s.

The results show that ab1A was the only class ORNs commonly activated by the three host fruits strawberry, raspberry and blueberry and by grape when only responses above 40 impulses/s were included. Each fruit activated a unique combination of at least two of these ORNs.

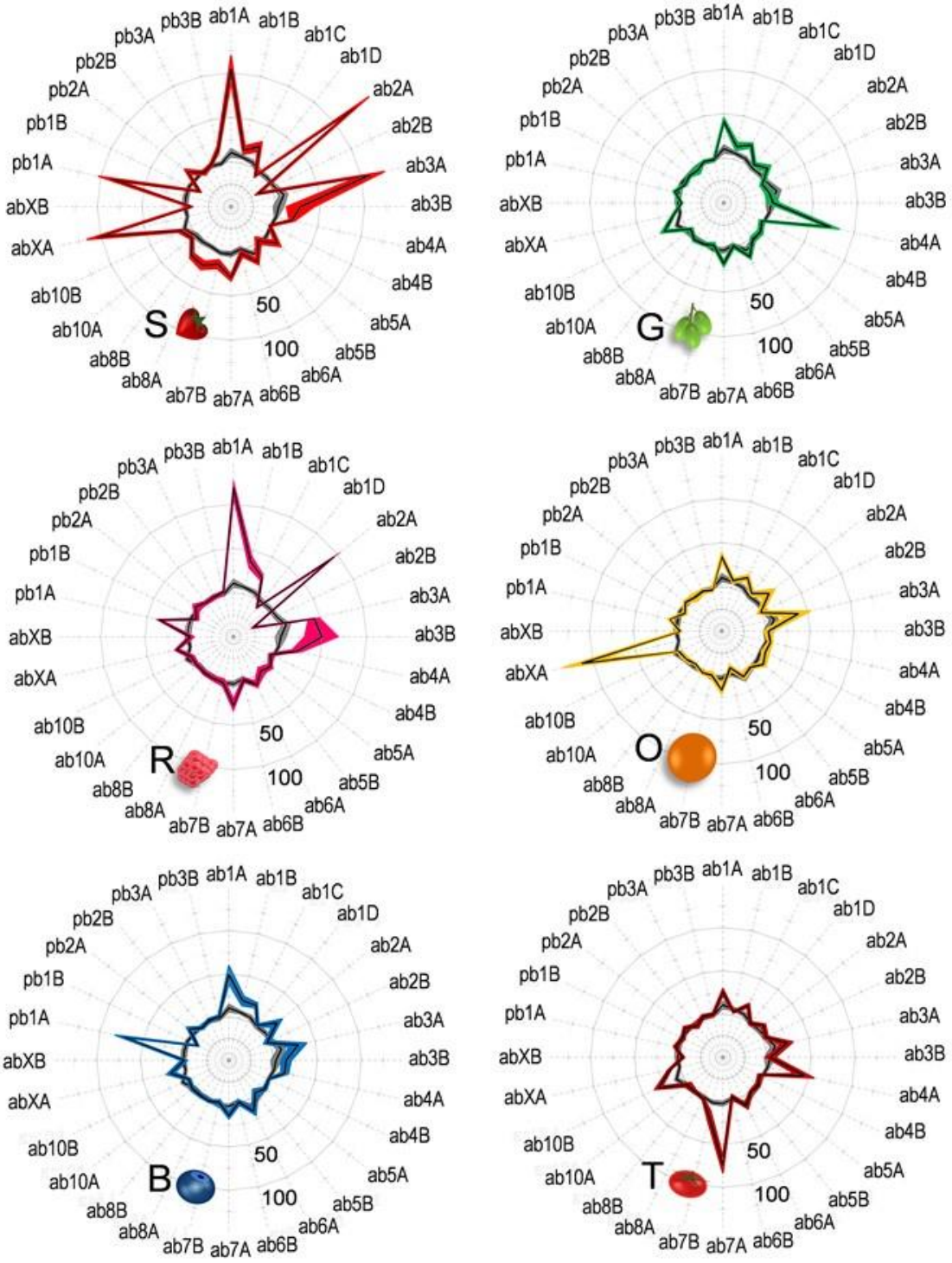


Figure 3-3 Fruitprints on the peripheral olfactory system in D. suzukii

Response rate during a 0.5 s stimulus of whole harvested ripe fruit headspace by the olfactory receptor neuron (ORNs) classes of the antenna (ab1A-abXB) and maxillary palps (pb1A-pb3B).Black lines and coloured shading show the mean response ± SEM for strawberry (S, red), raspberry (R, magenta), blueberry (B, blue), grape (G, green), tomato (T, dark red) and orange (orange). The response to ambient air is shown (grey) in each graph. Axis range from -50 (centre) to 150 impulses/s (periphery). N= 10-15 sensilla on 1-3 gravid females.

The activation of ab2B was unclear because of the high firing rate of ab2A by strawberry and raspberry. Their electrophysiological recordings were compared with the response to ethyl acetate, ligand for ab2A only (Figure 3-4). The ab2A neuron appear as the only one to be strongly activated by strawberry and raspberry headspaces. An activity of ab2B may still be existent for strawberry headspaces for which the response rate is higher, and the amplitude of the response appear stochastic compared to responses to raspberry and ethyl acetate, as shown in the traces below.

It can be concluded that ab2A respond to the two host fruit headspaces and that ab2B may be activated by strawberry headspaces. Until these activities can be separated with additional experiments, the ab2B is considered as not activated by these two fruit headspaces.

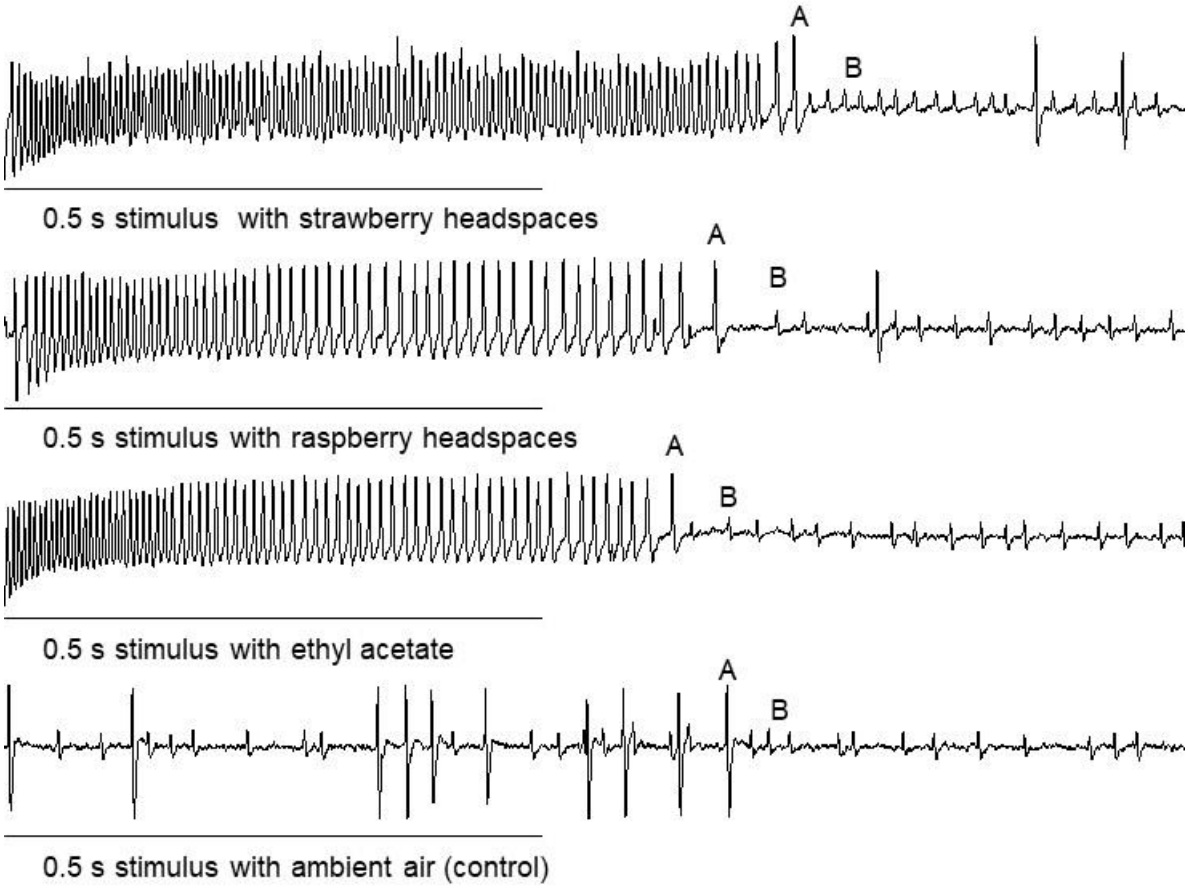


Figure 3-4 Traces of the ab2A and ab2B responses to strawberry and raspberry headspaces

Extracellular recordings in an ab2 sensillum following a 0.5 s stimulus with headspaces of strawberry, raspberry, control (ambient air) and ethyl acetate (30 µl of a 1% solution). The annotated action potentials A and B are from the ab2A and ab2B neurons respectively.

3.3.2 Only a subset of ORNs is activated by fruit odours

In order to determine which of these ORNs may be the most implicated for detection and selection of host fruits, the next step of the analysis aimed to characterise them as highly activated, activated or not activated. Classes of ORNs were ranked from the most activated to the least activated by fruit headspaces (Figure 3-5). With more than 50 impulses/s when stimulated with at least one of the fruit odours, the seven classes of ORNs ab1A, ab2A, ab3A, ab4A, ab7A, abXA and pb1A were the most activated by fruit odours. The ORNs were categorised by the similarity of their responses using a Hierarchical Cluster Analysis (HCA). It was constructed from the ORN responses to each fruit odour on orthogonal axes to see whether classes could be grouped into functionally similar clusters (Method, 2.8). The agglomeration schedule of the HCA suggests that a two-clusters solution is most reasonable (Figure 3-5). The seven classes of ORNs ab1A, ab2A, ab3A ab4A ab7A abXA and pb1A were grouped into one cluster and were highly activated by fruits. They formed a characteristic fruitprint and were called fruit activated ORNs.

To identify which classes of ORNs were activated or not by fruit odours among the ones grouped into the second cluster, the responses to fruit headspaces were compared to the control stimulus (ambient air) using Wilcoxon signed-ranked tests (Figure 3-5, Appendix 2). The types ab10A, pb3A, ab5B, ab1D, ab4B, ab6B, pb2B and abXB were not activated by any fruit headspaces as their response to fruit headspaces was not significantly different from the response to ambient air (control). Most of the other classes that were activated, only responded to one or two of the fruit headspaces with a small increase in response compared to control, below 40 impulses/s. Lastly, responses to fruit headspaces of the classes ab2B and pb1B were significantly decreased compared to the control (Figure 3-5, Appendix 2). However, the activity of these ORNs may have been masked by the higher response from their co-localised ORN (see above, Figure 3-3).

It can be concluded that not all classes of ORNs are involved in the detection of ripening fruits. The seven classes of ORNs ab1A, ab2A, ab3A ab4A ab7A abXA and pb1A are the most activated and used in further studies.

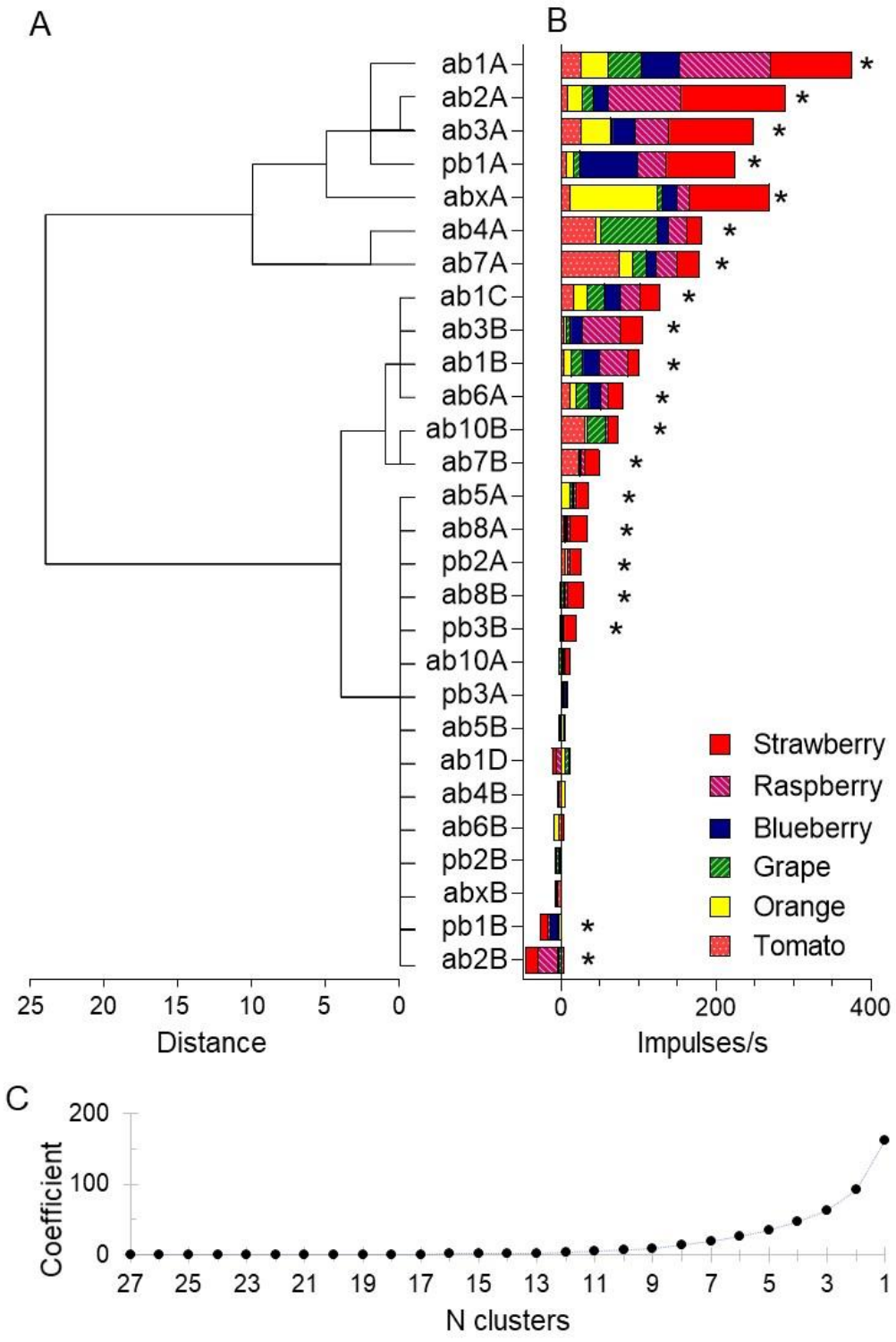


Figure 3-5 Classification of ORN responses to fruit headspaces

A) Agglomerative Hierarchical Cluster Analysis (HCA) using Ward's method on squared Euclidian distances. B) Stacked mean response rate to fruit headspaces for each ORN class. *Significant difference of at least one response to fruit headspace in comparison to control (stimuli with ambient air) using Wilcoxon signed-ranked tests (Appendix 2). C) Agglomeration schedule of the HCA suggesting a two-clusters solution: the distance coefficient between number of clusters is the largest between one and two clusters.

3.3.3 Fruitprints are plant species-specific

Differences in attractiveness to different cultivars was observed in *D. suzukii* (Introduction 1.1.2). Furthermore, the above fruitprints were determined using fruits from several cultivar of a plant species. To identify whether flies can discriminate among cultivars using their olfactory system, responses of ORNs on the antenna to different cultivars of strawberry and raspberry were measured.

Recording the activity of ORNs to headspaces of different cultivars showed that the same ORN classes were activated but at different relative intensities (Figure 3-6). The blueberry cultivar Ventura induced a significant lower response of ab3A, ab3B and ab1B. Similarly, the strawberry cultivar Winterstar induced significantly lower responses of ab1A, ab2A and ab4A compared to the two other cultivars (Appendix 3). The cultivar Cuna (strawberry) activated ab8A with more than 50 impulses/s. Due to the large variability of this response it was not significantly different from control or from the two other cultivars. It can be concluded that fruitprints are species-specific because the same classes of ORNs are activated but that cultivars may be differentiated by the intensity of activation of these ORNs.

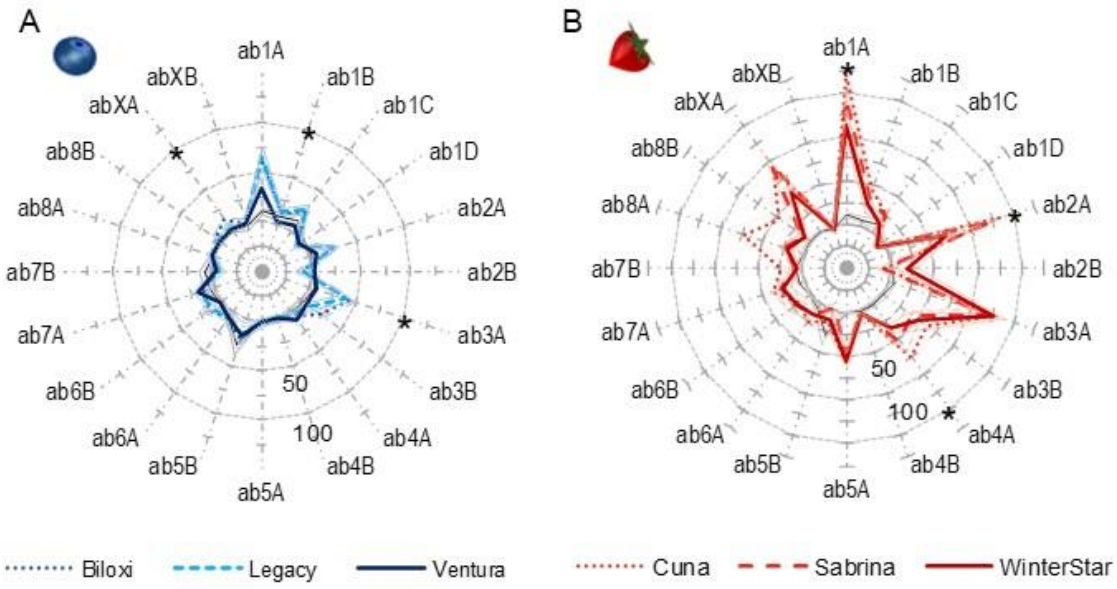


Figure 3-6 ORN responses to fruit headspaces of three cultivars of blueberry and strawberry

Mean response rates (impulses/s) from classes of ORNs on the antenna of *D. suzukii* to headspaces of three cultivars of Blueberry (A): Legacy (light blue), Biloxi (dots, light blue) and Ventura (dark blue); and Strawberry (B): Cuna (dots, dark red), Sabrina (light red) and Winterstar (dark red). No ab9 ORNs were recorded from. N=6. * Significant differences following a Wilcoxon signed-ranked test, $P<0.05$.

3.3.4 Fruitprints change with post-harvest aging

An additional observation on grape headspaces revealed that several days after purchase (and without refrigeration) the fruit headspaces did not activate ab4A. The volatile profile of grapes may have changed with aging of the fruit and is likely associated with decay. Indeed, some fruits will ripen further, others like grapes will start decaying after harvest (Abeles *et al.* 1992). Clusters of grapes were kept at room temperature and electrophysiological responses to headspaces were measured on the day of purchase and two days later. The ab4A which is the most activated class of ORNs by grape headspaces (Figure 3-3) was no longer activated after the fruits aged (Figure 3-7). This suggested that the headspace composition and their detection changed with post-harvest aging of grapes. It can be hypothesised that fruitprints reflected the ripening stage of the fruit and may be a mechanism of recognition of the ripeness of this fruit.

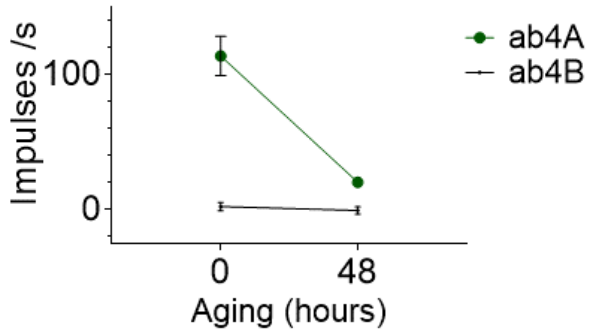


Figure 3-7 ab4A response to grape headspaces with post-harvest aging
Mean (± SEM) response rate of ab4A and ab4B neurons to grape headspaces on the day of purchase and 48h later, at room temperature. The date of harvest was unknown.

3.4 ATTRACTION TO HOST AND NON-HOST FRUIT HEADSPACES

The second objective of this chapter was to determine whether the fruitprints on the peripheral olfactory system can be associated with a behavioural response to fruit headspaces. The host status of the fruit used above was determined with oviposition assays. Then, attractiveness was assessed with long range and short-range behavioural assays, designed for gravid females *D. suzukii* (Method, 2.7; Appendix 1).

3.4.1 Three hosts and three non-host fruits

The susceptibility to oviposition of the six fruits used above was assessed in a no-choice oviposition assay with a single undamaged fruit available for 24 h. Females laid 5-7 eggs on strawberry and raspberry and 2-4 eggs on blueberry. Only one oviposition attempt was found on grape and none were observed on tomato and orange (Figure 3-8). It was concluded that strawberry, raspberry and blueberry are host fruits and grape, orange and tomato are non-hosts for *D. suzukii*.

Then, the behavioural response to headspaces was assessed in in a wind tunnel and 4-choice cage assay (Figure 3-8). In a wind tunnel: the long-range attraction to fruit headspaces was assessed by comparing the numbers of females on the upwind side of the wind tunnel where the headspace originated and 90 cm downwind where the flies started. The short range (the two baits were approximately 15 cm apart on the upwind side) attraction was assessed for the proportion of females which reached the upwind position by comparing the number of females that landed on the bait containing the fruits with the control bait containing water (Method 2.7). Preference indices (PI) were used to represent the dual choices (Figure 3-8). A 4-choice cage assay was used to assess the short-range attraction to fruit headspaces only without visual cues and without induced directional wind. The starting point was approximately 15 cm from each of the four baits in a cubic rearing cage (Method 2.7). The results were analysed with two statistical analysis, a sample t-test on the PI and General Linear Models (GLM) on the number of flies for both assays (Appendix 4).

The attraction to ripe fruit odours was very similar and the highest for strawberry and raspberry fruits followed by blueberry fruits in both behavioural assays. Strawberry and raspberry headspaces significantly attracted more females than controls in both behavioural assays. Blueberry headspaces significantly attracted more females in a wind tunnel but not in the 4-choice cage assay. Grape headspaces did not attract more females than controls in both assays, nonetheless the PI indicated a significant attraction to the fruit in a wind

tunnel. Tomato and orange headspaces attracted as many females as controls in both assays.

On the upwind side of the wind tunnel, visual cues were not discarded. All but orange fruits were preferred over water by attracted females. Less than 50% of females were attracted upwind by tomato headspace yet, nearly 80% of these preferred the tomato over water, when only 50% preferred the orange over the water in a wind tunnel. Control tests in both paradigms (all were water) showed that there was a random distribution of the flies in the absence of baits.

It can be concluded that strawberry and raspberry are host fruits which headspaces are the most attractive to gravid female *D. suzukii*. The host fruit blueberry is less attractive compared to strawberry and raspberry. Grape was less attractive and did not qualify as a host as it was not oviposited. The non-hosts orange and tomato were the least attractive in both long distance and short distance behavioural assays. In addition, it can be concluded that in vicinity, the colour red is attractive and visual cues may prevail.

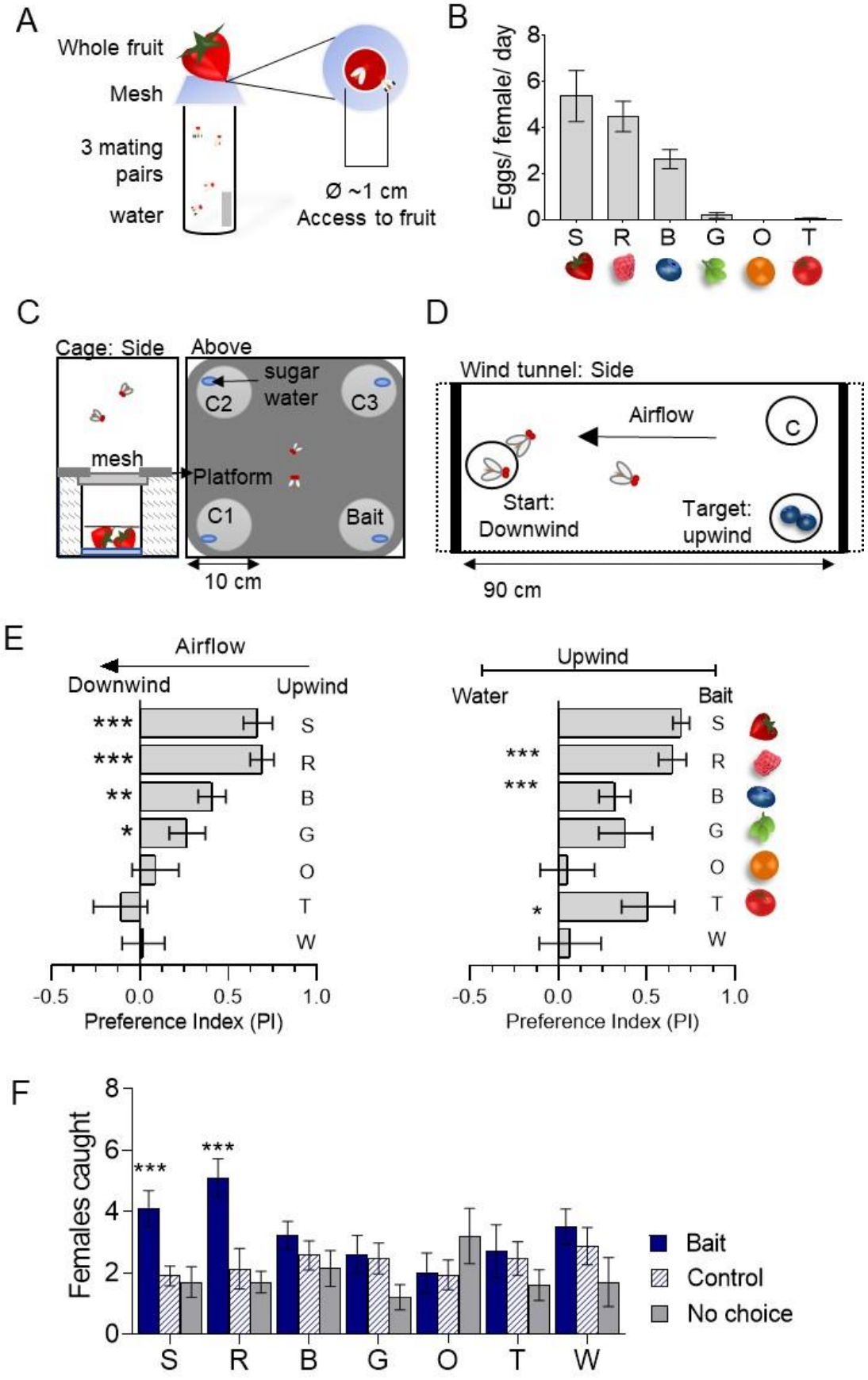

A
Whole fruit
Mesh
3 mating pairs
water
Ø ~1 cm
Access to fruit
B
Eggs/ female/ day
S R B G O T
C
Cage: Side
Above
sugar water
C2
C3
mesh
Platform
C1
Bait
10 cm
D
Wind tunnel: Side
Airflow
C
Start: Downwind
Target: upwind
90 cm
E
Airflow
Downwind
Upwind
S R B G O T W
Preference Index (PI)
Upwind
Water
Bait
Preference Index (PI)
F
Females caught
Bait
Control
No choice

Figure 3-8 Behavioural responses to host and non-host fruit volatiles

A) Oviposition assay. B) Mean (± SEM) number of eggs laid by females. C) Scheme of a 4-choice cage assay (CA) in which a fruit (bait) is presented against three controls (water). D. Scheme of a wind tunnel (WT) in which a fruit (bait) is presented against a control (water) upwind of an airflow. E) Mean (± SEM) preference index (PI) in a wind tunnel. PI= (N_{upwind}-$N_{downwind}$) / N_{total} (left) and for the females having reached the upwind position: PI = (N_{bait}-N_{water}) / N_{upwind} (right). N= 6-8. F) Mean (± SEM) number of females collected from bait, controls and no choice (which were not found on any bait) in a 4-choice cage assay. N=10-13. For all panels S" strawberry, "R" raspberry, "B" blueberry, "G" grape, "O" orange, "T" tomato, "W" water. Poisson GLM and Sample t-test, "***", P<0.001; "**", P<0.01; "*", P<0.05 (Appendix 4).

3.4.2 Host fruit preference

Preference among fruits was also assessed in order to investigate some modalities of host selection strategies employed by *D. suzukii*. Using a 4-choice cage assay three visible and accessible fruits and one control were simultaneously presented to gravid females in a rearing cage (Method 2.7). The fruits were ranked, raspberry being by far the most preferred with the highest attraction followed by blueberry and grape (Figure 3-9).

3.4.3 Role of olfaction and visual cues in attraction to host fruits

Then the preferences were assessed without visual cues. The three fruits and a control were presented in the 4-choice cage assay (Method 2.7) in which only headspaces could be detected by the fly. The attraction to raspberry was about 30% lower when the bait was not visible. Raspberry remained the most preferred with about 10% more females caught on the bait but that was not statistically different from the water bait. It can be concluded that females were not preferentially attracted to raspberry when their headspaces were simultaneously presented with blueberry and grape headspaces, in the absence of visual stimuli.

3.4.4 Host preference and effect of exposure

The hypothesis tested in this section is that gravid females are preferentially attracted by hosts to which they were pre-exposed to. Using the same fruits as above, flies were reared for more than 10 generations with an added fruit type to their diet: either raspberry, blueberry or grape. Their preference among the three fruits was then tested with visible and freely accessible fruits (Figure 3-9, Appendix 10.4). Raspberry was the preferred fruit, followed by blueberry and grape regardless the prior exposure. It was concluded that exposure to fruit prior to the experiment did not induce a change in preferences.

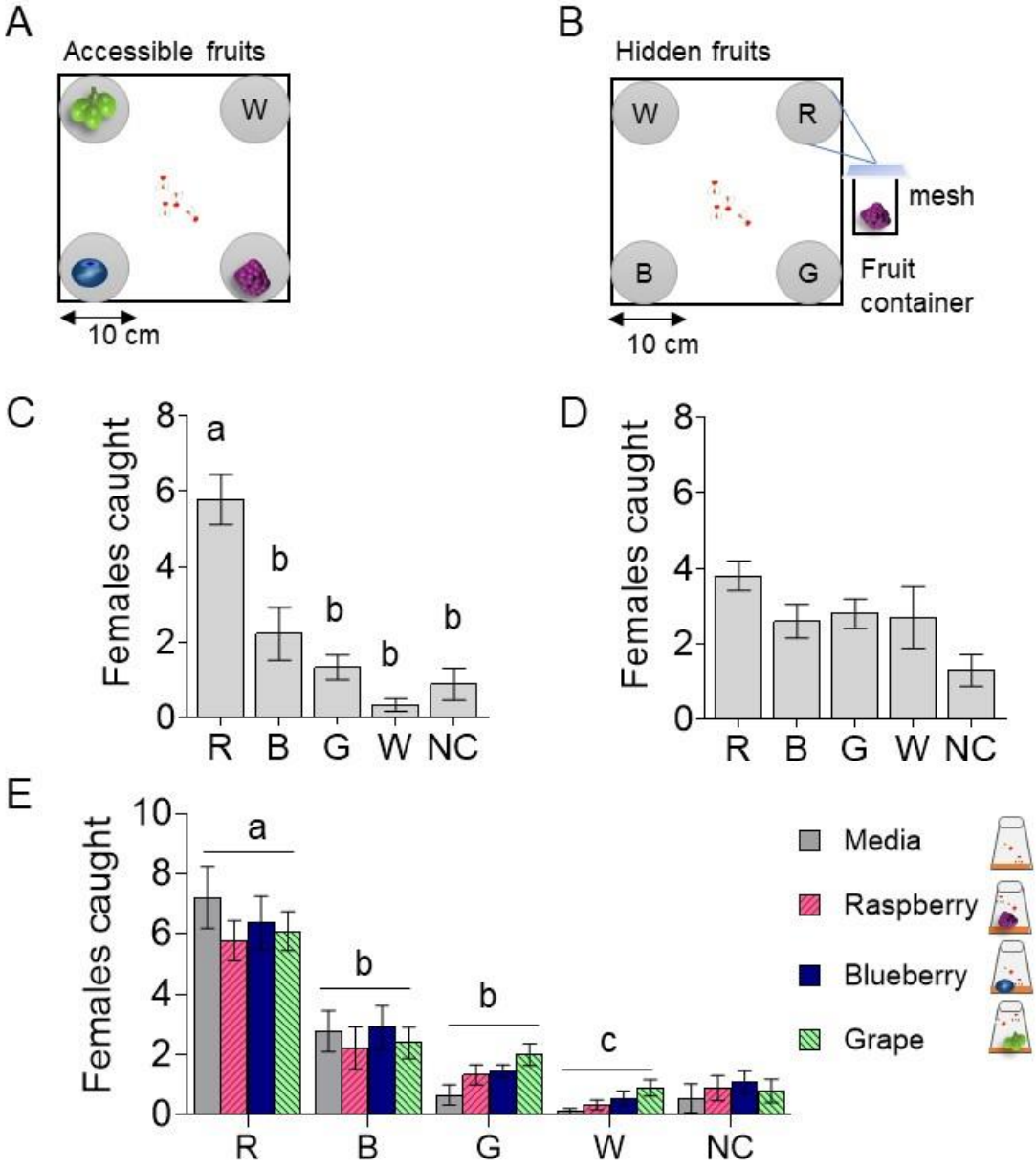


Figure 3-9. Host preference, visual and olfactory discrimination

Multiple choices assay with A) visible and accessible fruits in Petri dishes; and B) hidden fruits in containers. Mean (± SEM) number of females caught on baits and in the cage in a multiple-choice assay (C) and in a 4-choice cage assay (D). E) Mean (± SEM) number of females caught on baits after exposure to different fruits for several generations, in the multiple-choices assay. N= 8-10. Baits contained water (W), raspberry (R), blueberry (B), grape (G). No choice (NC) refer to females collected in the cage but not on any bait or control. Media refer to the food substrate used for rearing flies. For all panels, bars with different letters are significantly different following a GLM (Appendix 4).

3.5 DISCUSSION

How does the olfactory system permit to process complex signals through the background of environmental chemicals enabling the insect to find its host plants? In species which are attracted to several plant species, host selection requires either the ability to process multiple inputs enabling to discriminate among host substrates, or the ability to detect host signals that are shared by all hosts (Figure 1.6). Gravid female *D. suzukii* were used as a model to tackle these questions via electrophysiological and behavioural studies of their detection of fruit volatiles.

The activation of specific and overlapping subsets of neurons by each fruit headspace provides the neural basis by which females could discriminate among fruits. Multiple olfactory circuitries may drive host fruit selection. Both parts of the hypothesis presented earlier are true to some extent: 1) host fruits can be recognised by the activation of a common subset of ORNs, which differentiate them from non-hosts; 2) host selection involves the activation of combinations of ORNs which encode each fruit type specifically (Figure 3-10). Results and their implications are discussed below.

3.5.1 Not all classes of ORNs are recruited to encode fruit odours

The present study demonstrated that only a subset of ORNs is activated by different fruits. These seven ORNs appear sufficient to enable the discrimination of six ripe fruits in *D. suzukii* and *D. melanogaster* (Chapter 6). The quantification of the response to fruit odours by the 28 classes of ORNs demonstrated that a subset corresponding to 25% were the only ones involved in the discrimination of host versus non-host odours, being activated with a significantly higher intensity (more than 40 impulses/s increase from the spontaneous activity) compared to the other ORNs. In addition, some subsets were not activated at all by any of the fruit headspaces confirming that encoding of fruit odours does not involve the complete repertoire of ORNs. These results refute a hypothesis stating that all ORNs form a combinatorial pattern of activation with different intensities mediating the recognition of fruit odours. Using the larval olfactory system, Si *et al.* (2019) and Dweck *et al.* (2018) demonstrated that more than 90% of ORNs contributed to behaviourally active signals, by being all activated with different intensities. Furthermore, Dweck *et al.* (2018) identified that over 50% of all ORNs were activated, thus involved in the evaluation of fruit odours in the adult Drosophila. The responses to fruits were categorised by the number of chemicals from fruit headspaces that were detected by each ORN class (Dweck et al. 2016, 2018). Many of these classes responded to many individually presented fruit volatiles yet, only seven classes responded to the whole ripe fruit headspace in *D. suzukii* in this study, and in *D.*

melanogaster (Chapter 6). In field condition, the perception of host fruits includes a whole headspace and not the detection to individual compounds is changed when in mixture. The consideration of the whole headspace instead of the tuning of ORNs to individual compounds was thus necessary to deepen the understanding of the encoding of host odours by the olfactory system.

How much activation can be considered high enough to detect host fruit odours from a background of odours from the habitat? Another aspect to consider is how much activation is considered behaviourally relevant. It is important to consider the different methods used in several studies. A statistical difference from the control group, which was not different from the spontaneous activity, was used in the present study. Earlier studies considered a behaviourally relevant activation to be twice the spontaneous activity (Dweck *et al.* 2018). The characterisation of how much activity is needed to induce a behaviour remain to be deciphered as discussed in chapter 4.

Almost all classes of ORNs were activated by strawberry headspaces under the consideration that a significantly different response rate from the control is behaviourally significant. This threshold was similar to the one considered by Dweck *et al.* (2018) of approximately twice the spontaneous activity. Under these circumstances, the results support their study. This result was however valid for only strawberry headspaces, not for other fruits which headspaces were weaker (chapter 4). Furthermore, headspaces were collected in an enclosed space and are therefore more concentrated compared to field conditions, in both their study and the present. Strawberry headspaces may have been more concentrated compared to other fruits. Similar quantities of fruits were used but the surface of skin hence, the release surface of headspaces was likely very different.

The background noise may contribute to activating more ORNs, with smaller intensities (Cafaro 2016). Thus, fruit headspaces may induce a pattern of activation that is overlaid with an activation by background odours. The most activated ORNs may therefore enable to differentiate the odour plume from the background. The olfactory system in *D. melanogaster* is indeed capable of separating the relevant signals from background noise (Kadohisa and Wilson 2006). Therefore, the most activated classes would remain under field conditions that include a higher dilution of headspaces in the wind and a higher background noise, which is very little or non-existent in laboratory conditions. It can be hypothesised that the fruit headspaces activate combinations of these seven most activated classes of ORNs (above certain threshold of approximately 40 impulses/s in addition to the spontaneous activity), and this encoding mediates host selection in *D. suzukii*.

In addition, other classes of ORNs, not activated by fruit odours (i.e. the activity during a stimulus with fruit odours was not significantly different from a control) (Figure 3-4) and their innervated glomeruli have been found involved in other mechanisms such as predators (ab10B) or bacteria (ab4B) avoidance (Stensmyr *et al.* 2012; Ebrahim *et al.* 2015). This shows that these ORN may have another specific ecological role such as the detection of danger.

3.5.2 A combinatorial pattern of activation drives the discrimination of host and non-host fruits

D suzukii may not have specialised on a common characteristic shared by all hosts as discussed above. The present study shows that the polyphagous fly *D. suzukii* may have the ability to discriminate between various host plants for ovipositon, defending the hypothesis (2). This indicate that not one dedicated circuitry is involved but rather several combinatorial pattern of activation of ORNs. A pattern of activation (fruitprint), is specific to each fruit type showing that a combinatorial encoding of fruit odours enables gravid females *D. suzukii* to discriminate among various fruits host and non-host, for oviposition. Hence, not one but several oviposition driving olfactory circuitry exist, involving combinations of several but not all ORNs as discussed above. The following hypothesis can be drawn: the difference in attractiveness of the six fruit headspaces may come from the quality and number of classes of ORNs activated.

3.5.2.1 Combinations mediating attraction

Multiple combinations can mediate different attraction. For instance, ab1A may be activated simultaneously with one of the other host-activated ORNs: pb1A. This mediate a small attraction as observed for blueberry (discussed below). An additional activation of ab2A and /or ab3A may enhance this attraction, as seen for strawberry. The trio ab1A, ab2A and ab3A also induced a very high attraction to raspberry. This suggest that a recruitment of two or more ORN in addition to commonly activated ORNs (here ab1A) may induce different behavioural responses. It also suggests that multiple combinations may induce attraction. For instance, the lack of attractiveness of blueberry headspace may be associated with the non-activation of ab2A and ab3A. These ORNs can indeed be confidently associated with attractive host fruits. The ab2 are the most represented ORNs on the antenna (see above) and are tuned to attractive volatiles such as ethyl acetate or isoamyl acetate (Mansourian *et al.* 2019; Revadi *et al.* 2015). Similarly, ab3A appear associated with the detection of ripening fruits in *D. suzukii* notably via detection of β-cyclocitral (Keesey *et al.* 2015).

3.5.2.2 Combinations preventing attraction

It can be hypothesised that combinations of attraction mediating ORNs types and non-attraction mediating ORNs types would induce attraction or avoidance depending on a balance between the number of ORNs activated from each type. The non-attractiveness of non-host may therefore be associated with the activation of the classes abXA, ab4A and ab7A in addition to a smaller number of host-activated classes of ORNs.

Indeed, ab4A and ab7A were only activated by the grape and tomato headspaces. These two were not suitable for egg laying, and their headspaces were not attractive in behavioural assays. A decrease in activation of ab4A with age was observed for grapes. It enables to hypothesised that the decrease in activation of ab4A may be associated with increased suitability of the fruit as it decays. For instance, sweetness may increase, and the skin may be prone to damages and be weakened and more accessible to female's ovipositor (Ioriatti *et al.* 2015). Further behavioural and electrophysiological experimentation would be required to test how the activation of ab4A and/or ab7A may prevent fruits from being attractive.

The ORN abXA is commonly activated by strawberry and orange headspaces and is therefore difficult to associate to a specific role. A common activation with host associated ORNs by strawberry headspaces indicate that the combination of four host activated ORNs cannot be inhibited by the activation of abXA. On the contrary, orange headspaces activated ab1A and ab3A with a lower impulse rate which may explain a non-attraction. Additional characterisation of abX and its role is needed.

3.5.3 Host and non-host activated ORNs

These results show that females can process multiple attractive signals and discriminate among host substrates. It is therefore unlikely that all hosts are recognised as one category. As discussed above, several classes of ORNs appear associated with dedicated behaviours such as attraction and repellence. In this model, the attractive substrate may activate several specific and ubiquitously tuned classes of ORNs. The present study suggests that host selection in *D. suzukii* is not mediated by a single olfactory circuit and is supported by Si *et al.* (2019) and Dweck *et al.* (2018) (as above). However, a host versus non host discrimination might take place.

3.5.3.1 Hosts activate common classes.

In this study, classes of ORNs appear subdivided into "host activated", which activation may induce attraction to the fruit substrate, and "nonhost" activated, for which no behavioural response was observed (Figure 3-10) suggesting an underlying specialisation. As described

in *D. melanogaster* classes of ORNs can be classified depending which behaviour they induce. For instance, ORNs and glomeruli were classified by three terms: the good, bad and hungry (Sachse and Beshel 2016) by their responses to mates (good), predators (bad) and food odours (hungry), and additional dedicated ORNs were linked to behaviours (Sachse and Beshel 2016; Haverkamp *et al.* 2018). Glomeruli were associated with attractiveness or repellency (Knaden *et al.* 2012; Mohamed *et al.* 2019) and the lateral horn (LN) is subdivided in regions tuned to attractive or repellent odour (Strutz *et al.* 2014). However, the study also demonstrates overlapping between these activations. The characterisation of fruit headspaces is needed to identify whether common volatiles shared by all hosts that females may detect and use for host selection.

Overall, ab3A and ab1A appear to have dedicated roles associated with attraction to host fruits. They may be more involved than any others in the adaptation to ripening fruits in *D. suzukii*. Indeed, orange is an attractive host to *D. melanogaster* but not to *D. suzukii* (Dweck *et al.* 2013). A comparative study with *D. melanogaster* supported a change in sensitivity to orange headspaces of these two ORNs in the *D. suzukii* lineage (Chapter 6). The role of ab3A in host attractiveness for *D. suzukii* is shown in this study and earlier. Changes in the function of ab3A in *D. suzukii* compared to *D. melanogaster* were demonstrated in chapter 6 and by (Keesey *et al.* 2015; Keesey *et al.* 2019). These supported changes in the expression of ab3 associated or22a (Ometto *et al.* 2013; Hickner *et al.* 2016; Ramasamy *et al.* 2016). Earlier studies stated that ab3A may drive host specialisation. Indeed, ab3A was identified as inducing attractiveness of flies and associated with a host shift in *D. suzukii* (Keesey *et al.* 2015) and other Drosophilids, including *D. melanogaster* (Mansourian *et al.* 2018), *D. orena* (Comeault *et al.* 2017), *D. sechellia* (Dekker *et al.* 2006) and *D. erecta* (Stensmyr *et al.* 2003).

The present study demonstrates that these classes are not the sole driver of host selection and therefore, that not all ecological roles may be encoded by the olfactory system following a single circuitry mechanism. Host selection seems to involve multiple classes of ORNs, which combinatorial patterns are fruit specific.

3.5.3.2 The ab1A mediates attraction

The ab1A neuron was commonly activated by the three hosts with high intensity (Figure 3-3). Given that each fruitprint was a combination of several classes of ORNs activated with the same intensity as ab1A, it is unlikely that this class is the only one mediating host selection. However, additional supporting evidence of its sufficiency to mediate attraction is required because the association of ab1A with attraction to the three host fruits provides support for the following statement: a single class of ORNs has a dedicated function. This

hypothesis was defended in earlier studies by (Hansson and Christensen 1999; Hansson and Stensmyr 2011; Sachse and Beshel 2016). A dedicated olfactory circuitry (i.e. ORN class) may induce host selection behaviour in species such as *D. melanogaster* which oviposition sites are narrowed to few olfactory cues related to fermenting processes by yeast and bacteria. For instance, oviposition was driven by Or19a (ai2A) by detection of the terpene limonene from citrus fruits (Dweck *et al.* 2013). Host specialisation was also hypothesised to be mediated by a single ORNs, the ab3A in specialist *Drosophila* (Dekker *et al.* 2006; Linz *et al.* 2013; Mansourian *et al.* 2018).

3.5.4 Intensity of activation and threshold

The intensity of activation of ab1A varied with the fruit headspace and was the highest for the most attractive fruits and the lowest for the least attractive fruits (Figure 3-3). This would imply that no threshold of activation is necessary, but a gradient of activation induce a gradient of behavioural response. The presence and intensity of activation to induce a behavioural response (or threshold) is not known and require further research (introduction 1.3). It is therefore possible that the attraction to strawberry and raspberry was associated with the higher activity of ab1A. Nonetheless this class of ORNs was not the only one activated by host fruit with high intensity suggesting that it is not the sole class of ORNs involved in the recognition of host fruits. Therefore, additional studies are needed to demonstrate whether the activation of ab1A is necessary and sufficient.

The comparison of three blueberry cultivars showed that the intensity of activation of ab2A and ab3A was significantly changed, for one of these cultivars. The limited attractiveness of blueberry in this study could thus be due to a cultivar variability in the fruit headspace composition. The same classes of ORNs were highly activated, regardless of the cultivar for both blueberry and strawberry, suggesting that fruitprints are species-specific. Differences in the intensity of activation of the host activated ORNs may thus also drive differences in attractiveness between cultivars. Indeed, blueberry cultivars were found to differ in terms of attractiveness and suitability by several field studies (Lee *et al.* 2016; Kinjo *et al.* 2013; Rogriguez *et al.* 2018). This result is supported by reports of the chemical composition of several cultivars of fruits in studies of their aromas, as for instance in raspberry, for which cultivars differed by the ratios of components rather by their nature (Larsen *et al.* 1991). It is therefore likely that the amount of chemicals produced by the blueberry used in this study was not as high as when on the plant. Other plant material volatiles were not included, and their role is further discussed in chapter 4. Lastly, post-harvest treatments and aging of the fruit greatly impact fruit volatile bouquets (De Ancos *et*

al. 2000; Forney *et al.* 2000; Vilanova *et al.* 2007). The latter was also demonstrated with the response to grape headspace via the ab4A ORNs.

3.5.5 Numbers of functional classes of ORNs illustrate a change in olfactory specialisation

Each of the 28 classes of ORNs housed in thirteen basiconic sensillum types could be recognised using a unique ligand from the diagnostic panel. This allowed a rapid identification of sensillum types before proceeding with further experiments. Descriptions in *D. melanogaster* were made showing a similar distribution of basiconic types on the third antennal segment (De Bruyne *et al.* 2001; Grabe *et al.* 2016).

ORNs housed in trichoid sensillum types, mainly involved in con-specific odour recognition, and coeloconic types, tuned to amines were not addressed here but should be considered in further studies. Indeed, attraction to substrates is enhanced by the association between food odours, nutrients and conspecific odours in *D. melanogaster* (Duménil *et al.* 2016; Gorter *et al.* 2016).

3.5.5.1 The ab2 ORNs are the most represented on the antenna in D. suzukii

The proportions of sensillum types were however different. In *D. suzukii* there is a larger proportion of ab2 and a smaller proportion of ab3 compared to *D. melanogaster*. These observations are in accordance with the results of Keesey *et al.* (2019). ORNs ab2A and ab3A were associated with host shift from Overripe to ripening fruits in *D. suzukii* (Abraham *et al.* 2015; Keesey *et al.* 2015; Hickner *et al.* 2016). In this study, both classes of ORNs were strongly activated by attractive ripe fruits suggesting they specialised on number of cues that are shared by ripe host fruits. However, the activation of ab2A was not necessary to induce attraction in a separate study (chapter 4).

3.5.5.2 The role of ab2B

The ab2B can be hypothesised to be activated by host odours. It may have been activated by fruit headspaces and masked because of the high firing rate of ab2A. The small amplitude action potentials of ab2B may have been easily overlooked for strawberry and raspberry headspaces (Figure 3-4). Strawberry and raspberry headspace also contain many volatiles that are ligands for ab2B (Chapter 4), suggesting its activation by the whole fruit odour.

Another hypothesis is that ab2B is inhibited by the high firing rate of ab2A, when exposed to the whole fruit headspace. It is known that the response to single components and their mixture may strongly differ (Laing *et al.* 1984; Silbering and Galizia 2007). Indeed, the ab2B impulse rate was significantly lower than the control indicating that its activity was either

completely masked or inhibited by ab2A. Modulation of signals is possible between co-localised ORNs (Dobritsa *et al.* 2003; Goldman *et al.* 2005; Su *et al.* 2012; van der Goes van Naters 2013) or postsynaptic partners via PNs (Gorur-Shandilya *et al.* 2017), and could be happening between the two ab2 ORNs. Lastly, ab2B innervates both DM3 and DM5 glomeruli, which are associated with attraction and aversion, respectively (Laissue and Vosshall 2008; Semmelhack and Wang 2009). A double mechanism associated with the activation of ab2B can therefore be hypothesised as discussed chapter 4 but remain to be tested.

3.5.6 Multimodal strategies of host selection

3.5.6.1 Host preference/ranking as a host strategy

Hosts for *D. suzukii* ripen one after the other thorough the year. It could be hypothesised that flies may become attracted to hosts as they become available if they developed ranked preference (Introduction, 1.2.4). Raspberry was the most attractive and most preferred followed by blueberry and grape by gravid female *D. suzukii*. The same ranking of hosts was consistently observed in several experiments and was previously assessed in different conditions (Abraham *et al.* 2015). This study further show that the preference ranking was maintained irrespective of prior exposure, which demonstrate that previous experience was apparently not determinant of female *D. suzukii* attraction to fruits. The ranking of fruits observed may thus be innate (Kadow 2019).

3.5.6.2 Visual, anemo and olfactory cues are combined for host selection

The absence of a directional wind in the 4-choice cage assay may have rendered the detection of odour plumes from the baits more difficult for the females as supported by a decrease in the number of females attracted by strawberry and raspberry. Wind direction is detected by dedicated sensory pathways which allow *D. melanogaster* flies to orient towards the source of an odour plume (Suver *et al.* 2019). An absence of wind may therefore render the olfactory orientation difficult in this species and related such as *D. suzukii*. Observation of their behaviour in the different assays also indicated that they mostly take off and fly for a few minutes rather than walking like *D. melanogaster* do which supports the theory that the detection the physical aspects of the odour plume (in addition to chemicals) may be decisive in this species.

This study also demonstrate that visual cues are very important but not sufficient for host selection in *D. suzukii*. In a short-range assay without visual cues females did not discriminate between raspberry blueberry and grape suggesting that in a background of several potential host odours, females are unable to find the most attractive host without

visual input. Furthermore, without olfactory cues a tomato was very attractive to females. This was shown in the wind tunnel, when from minority of females which reached the upwind side, 80% landed on the tomato in which they do not lay eggs. By comparison, orange fruits were not preferred over control in the same conditions. Olfactory cues therefore permit to discriminate between suitable and non-suitable fruits for oviposition when the fruits share similar visually attractive features (such as a big red ball). The role of visual cues is further supported by a recent study showing the increased size of the visual system in *D. suzukii* compared to *D. melanogaster* (Keesey *et al.* 2019). In addition, the development of traps to monitor and manage populations in commercial crops showed that traps designed for visual attractiveness and are the most efficient when associated with olfactory attractive baits. For instance, red and black striped traps are so far the most efficient at capturing *D. suzukii* compared to transparent traps (Basoalto *et al.* 2013; Lee *et al.* 2013; Grassi *et al.* 2014; Renkema *et al.* 2014). A red and black trap, associated with a specific bait was also the most efficient tested against *D. suzukii* in field trials (Cloonan *et al.* 2018; Kirkpatrick *et al.* 2018). Lastly, red traps are the most visually competitive with the fruits when surrounded by yellow fruits (such as Guava) (Basoalto *et al.* 2013). Different traps and baits are regularly tested and improved in heavily threatened cherry orchards in Italy (pers. observation and communication, A. Grassi).

3.5.7 Insights on the behavioural response to olfactory cues

The 4-choice cage assay and wind tunnel are good paradigms to test *D. suzukii*, because they allow flight and enough time so more than 80% of the tested population respond to the test. The development of the assay in this thesis showed that travel in *D. suzukii* adults appears to involve several flight periods lasting a few minutes and large amounts of time without moving (Pers. observation) unlike *D. melanogaster* which explores its near environment walking. It was concluded that the successes of behavioural assays are mainly due to giving the flies the space and time needed to forage. In addition, the possibility to land on the bait rather than having to walk down a narrowed trap entry was associated with higher response rates (appendix 1). For instance, less than 50% of females responding in Multiple choice assays with trap entries (chapter 5).

Depriving the females from food and oviposition substrate prior testing enhanced the foraging activity (Appendix 1). The disadvantage was that starvation may have occurred which drove females to forage for food instead of oviposition substrate (Dethier, 1976). However, starvation was also shown to increase the response rate in behavioural assays, without changing the outcome in *D. melanogaster* (Becher *et al.* 2010). Females would search for oviposition substrate when fully fed as shown by Clymans *et al.* (2019). They

confirmed that the physiological state of *D. suzukii* induced significant changes in their behavioural response to food odours. Providing the flies with a food source (arrest) on each bait permitted to reduce the likelihood that the tested bait was chosen because it represented a food source, instead of an oviposition substrate. However, the attraction to oviposition substrate may have been compromised by hunger and thus be underestimated.

Physiology (e.g. hunger) and state such as sex or mating status have been shown to influence the insect's response to food and conspecific odours (Hern and Dorn 1999; Datta *et al.* 2008; Barrozo *et al.* 2010). For instance, mated insects no longer seek mates but oviposition substrate or food (Mechaber *et al.* 2002) The motivational state of the flies along with test conditions (i.e. laboratory assays) also modulates the olfactory response in controlled environment. For instance, an increased sensitivity to food odours was indeed caused by starvation in *D. melanogaster* (Ko *et al.* 2015; Cao *et al.* 2014). The detection of CO_2 is mediated by two different pathways leading to attraction in foraging flies and indifference in resting flies (van Breugel *et al.* 2018)

3.6 CONCLUSION AND FURTHER DIRECTION

The results of this chapter demonstrate that the peripheral olfactory system in *D. suzukii* encodes fruit odours as follows: each fruit headspace evoke a pattern of activation from seven classes of ORNs. These fruitprints are plant species-specific showing that gravid females can discriminate among host fruits (Figure 3-10).

Additional research is needed to fully characterise how the encoding of fruit odours drives host selection. The following questions are addressed in the next chapter: Which fruit volatiles are detected by gravid female and perceived as attractive host cues? Which combinations of classes of ORNs mediate attraction to host fruits?

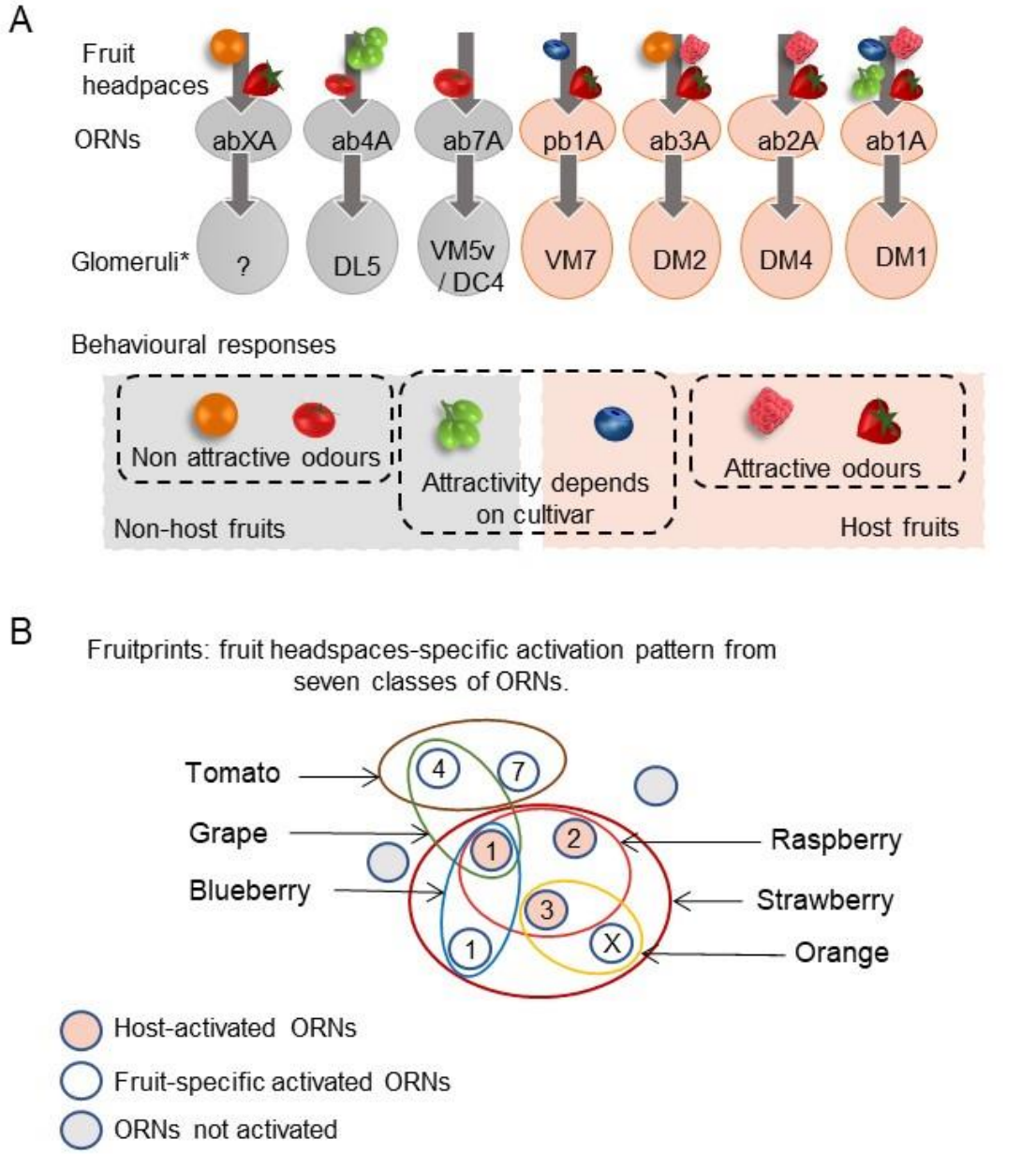


Figure 3-10 Fruit headspaces activate specific and overlapping ORNs

A) Seven classes of ORNs were the most activated by the three host fruit types: strawberry, raspberry and blueberry and the three non-host fruit types grape orange and tomato. Activations above 40 impulses/s were considered. The activation of the classes of ORNs pb1A, ab3A ab2A and ab1A appear correlated to the attraction of the gravid females for oviposition and are called host activated. ORNs innervated specific glomeruli under the assumption that the innervation on the antennal lobe is similar as in *D. melanogaster* (Introduction, 1.3.1). The type abXA was not previously characterised. Blueberry and grape displayed variability which may be due to cultivar. B) Seven overlapping and specific classes of ORNs were activated by the ripe fruit headspaces. Filled circles represent the characterised classes ab1A, ab2A, ab3A, ab4A abXA, ab7A and pb1A (only the number is shown). The three firsts (pink) were commonly activated by the two most attractive headspaces of strawberry.

CHAPTER 4

4 IDENTIFICATION OF FRUITSEMIOCHEMICALS AND PROSPECTS FOR PEST MANAGEMENT

Female *Drosophila suzukii* on a blueberry, with a raspberry in the background. Rothamsted Research ©.

4 IDENTIFICATION OF FRUIT SEMIOCHEMICALS AND PROSPECTS FOR PEST MANAGEMENT

4.1 INTRODUCTION

4.1.1 Aim of chapter

Polyphagous insects such as *D. suzukii* must be able to recognise taxonomically distant hosts. The previous chapter of this thesis demonstrated that host and non-host fruit headspaces are encoded by subsets from seven classes of olfactory receptor neurons (ORNs). The responses to fruit headspaces can be characterised by specific fruitprints or patterns of activation of these ORNs. Aspects of these encodings remained unclear, notably the composition of fruit headspaces and how these fruitprints mediate attraction to fruits.

The aim of this chapter is to identify fruit volatiles that are encoded by fruit-activated classes of ORNs in *D. suzukii* and to identify combinations of ORNs which mediate attraction similar to one of the host fruits. Following the previous chapter (Chapter 3), the following hypotheses are addressed: A) host fruits are selected via the detection of specific and shared volatiles, enabling the female to discriminate between multiple hosts; B) more than one class of ORNs is necessary to induce attraction to host fruits (Figure 1-6). Furthermore, semiochemicals that activated the same olfactory circuitries as host fruits are candidate attractants which can be tested in behavioural assays. Attractive or masking/repellent volatiles may be used to develop novel baits for sustainable management of *D. suzukii* in fruit orchards.

Headspaces of six ripening fruits (hosts and non-hosts), whose fruitprints on the peripheral olfactory system in *D. suzukii* have been determined (Chapter 3), were collected and analysed using electrophysiology and analytical chemistry. Harvested whole ripe fruits were used in order to obtain volatile profiles corresponding to fruits which skin is undamaged as when ripening on the plant (Introduction, 1.1.2). Extracellular recordings on the seven sensillum types housing fruit activated ORNs were done with compounds identified in the headspaces. This allowed the key volatiles that contributed to a fruitprint to be identified. The behavioural effect of activating subsets of ORNs was tested to identify olfactory circuitries mediating attraction to host fruits.

Gravid female *D. suzukii* mostly detected fruit-specific volatiles. Few of the bioactive volatiles were shared by two or more fruits, and none of them were shared by all three host fruits. In addition, results suggest that several combinations of two or more fruit activated ORNs mediate attraction. The recognition of all hosts by *D. suzukii* did not appear mediated

by a single olfactory circuitry. Based on which classes of ORNs were activated by these fruit volatiles, the behavioural valence of each compound can be hypothesised.

4.1.2 Background

In many species the first step in host selection relies on olfactory cues enabling the insect to detect and orient towards potential hosts. Plant odours are processed via a highly sensitive and specialised olfactory system (Visser 1986; Visser 1988; Hansson *et al.* 1999; Schoonhoven *et al.* 2005; Bernays and Chapman 2007).

4.1.2.1 Plant semiochemicals

Plant volatiles may carry key informative cues to select hosts for food, oviposition, mates or to avoid danger (e.g. toxicity, predators). Each insect species detects a unique set of these semiochemicals. Some are relevant to food foraging and others are associated with searching mates (Price *et al.* 1980; Dicke 2000; Reddy and Guerrero 2004). Chemicals can also overlap and interact when presented together (Introduction 1.2) indicating that multiple messages may be conveyed by semiochemicals depending on their ratios in mixture.

Strict specialist phytophagous insects are often attracted to their host via the processing of a single specific compound or a specific mixture of compounds it releases (Dicke 2000; Bernays and Chapman 2007). On the contrary, generalist insects must be able to recognise several hosts which released chemical bouquet include a broader range of ubiquitous and specific compounds. How they use semiochemicals for host selection is unclear. Perhaps chemicals or mixture of chemicals may be shared by all potential hosts, suggesting a level of specialisation (Bolnick *et al.* 2002). Perhaps they can discriminate and recognise each host type and develop preferences (Cardé and Willis 2008). Preferences may be a first step in specialisation to subsets of hosts from the range sustained by generalist insects (Bernays 1998; Bolnick *et al.* 2002).

The polyphagous fly *D. suzukii* is attracted to many distinct fruit types and can be used as a model to study the discrimination of multiple hosts. In addition, the identification of host selection mechanism in this species may inform pest management. A deeper understanding of host-insect interaction would enable to design species-specific control tools.

4.1.2.2 The peripheral olfactory system

The olfactory system in *D. suzukii* can be briefly summarised as follows. It consists of olfactory receptor neurons (ORNs) that are housed in sensilla, which are finger-like or hair-like cuticular structures on the surface of the antennae and maxillary palps. ORNs harbouring the olfactory receptors (ORs) tuned to few or many different food and host volatiles in their dendrites are assembled into functional basiconic sensillum types

(Introduction, 1.3). Approximately 13 functional types of basiconic sensillum labelled ab1 through ab10 are on the third antennal segment and pb1, pb2 and pb3 are on the maxillary palp. The ORNs can be recognised by the amplitude of their action potentials on electrographs (Method 2.8.2). For instance, the basiconic sensillum type ab1 houses four ORNs: ab1A, ab1B, ab1C and ab1D. These ORNs relay olfactory information towards the glomeruli of the antennal lobe (Introduction 1.3).

Extracellular recordings (SSR) of the activity of these ORNs allow their response to chemical stimuli to be measured (Kaissling 1995). This technique permitted to characterise the classes of ORNs in *D. suzukii* (chapter 3). Furthermore, the recording of the activity of these ORNs in response to ripe fruit odours permitted to identify that seven of these classes of ORNs encode ripe fruit odours in *D. suzukii* (Chapter 3). These activations were characterised as strong and relevant with more than 40 impulses/s during a stimulus (Chapter 3).

4.1.2.3 Encoding of food/ host odours

ORs specificity can be characterised by their response profiles to hundreds of different chemicals (Mathew *et al.* 2013; Münch and Galizia 2016). Thousands of different odorant compounds have been tested on the several ORNs and their receptors in *D. melanogaster*, and much of these data have been standardized into the freely accessible database DoOR (Münch and Galizia 2016). Fruit volatiles belong to various chemical classes that are detected by ORN classes including for instance esters, alcohols, aldehydes, ketones, aromatics and terpenes.

The antennal lobe can be regionalised. ORNs and glomeruli were found to be specific to the valence of volatiles such as attraction and repellency (Grabe *et al.* 2016; Sachse and Beshel 2016). Some innervating ORNs have also been associated with ecological roles as mate finding or predator avoidance (Kurtovic *et al.* 2007; Knaden *et al.* 2012; Mansourian *et al.* 2016) and can be tuned to a few chemical classes and similar structures, as demonstrated with alcohol (Bichão *et al.* 2005; Si *et al.* 2019).

The specificity of ORs is also a function of odour dose: some ORs are tuned to specific odours but can become tuned to a broader range of chemicals at high doses. Indeed, some ORs can be strongly activated by many odours at low doses while other ORs have a narrow window of specificity even at high doses (Kreher *et al.* 2008). Higher doses of chemicals induce the recruitment of additional ORNs and glomeruli, which may change the behavioural responses as for instance, turn attractive chemicals into repellent (Malnic *et al.* 1999; Stensmyr *et al.* 2003; Wang *et al.* 2003; Semmelhack and Wang 2009). For instance, (*R*)-carvone activated the same glomeruli DM1 (hence, likely mediated via ab1A/ Or42b) as

its enantiomer when tested at a dose of 5 % instead of 2 %. Furthermore, nearly twice the number of glomeruli were activated when the dose of a same chemical was doubled (Wang *et al.* 2003).

Furthermore, if ORs have multiple binding sites, the binding strength of the odour molecule to the ORs depend on how each site contribute to the binding. This was proposed by lancet et al (1993) and supported by Si et al (2019). It suggests that the affinity of the OR is dependent on how many molecules it can bind. The OR-ORCO complex that has a tetrameric form with four subunits (Butterwick *et al.* 2018) may enable these differences in affinity, if each subunit permit the binding of odorant molecules.

4.1.2.4 Detection of mixtures

VOCs from several chemical classes are found in plants headspaces. A host originating blend may therefore simultaneously activate several of ORNs and glomeruli in ratios that depends on the chemical classes content. The detection of different optical isomers of an odorant molecule may also be critical, because two enantiomers with the same structure might have different ecological roles. Enantio-specific ORs are found in several species. An example is the mosquito *Aedes aegypti* olfactory receptor AaOR8, which responds to the (*R*)-1-octen-3-ol but not to the (*S*)-1-octen-3-ol form (Bohbot and Dickens 2009). This enantio-selectivity was accompanied by a differential behavioural outcome: (*R*)-1-octen-3-ol was attractive to *A. aegypti* and *Anopheles gambiae*, but the (*S*)-form was repellent to *Culex quinquefasciatus* detected via the enantio-selective CquiOR114b (Xu *et al.* 2015). In *D. melanogaster*, evidence of enantioselectivity was found with calcium imaging of odour-evoked response of glomeruli in the AL in *D. melanogaster*. Wang et colleagues (2003) found different glomeruli to be activated following stimulation with (*R*)-carvone (DP1m) and (*S*)-carvone (DP1m and DM1) (Wang *et al.* 2003). DM1 is innervated by the ORN ab1A expressing Or42b (Laissue and Vosshall 2008). Hence, ab1A might be enantiospecific to the (*S*)-carvone as it did not respond to (*R*)-carvone.

4.1.2.5 Monitoring and management using semiochemicals

The use of semiochemicals as part of IPM strategies is crucial to monitor and control pest populations. It was shown to be efficient for disruption of signals, notably of sexual communication in pest *Lepidoptera* (El-Sayed *et al.* 2006; Reddy and Guerrero 2010). Plant originating semiochemicals that have been associated with attractiveness or repellency of insects can be exploited to disrupt the attraction of these to valuable crop in many systems (Pickett *et al.* 1997; Pickett and Khan 2016; Nishida 2018; Schlaeger *et al.* 2018). They can be used to manipulate behaviours, so insect pests are repelled from host plants or preferentially attracted to artificial lures in traps.

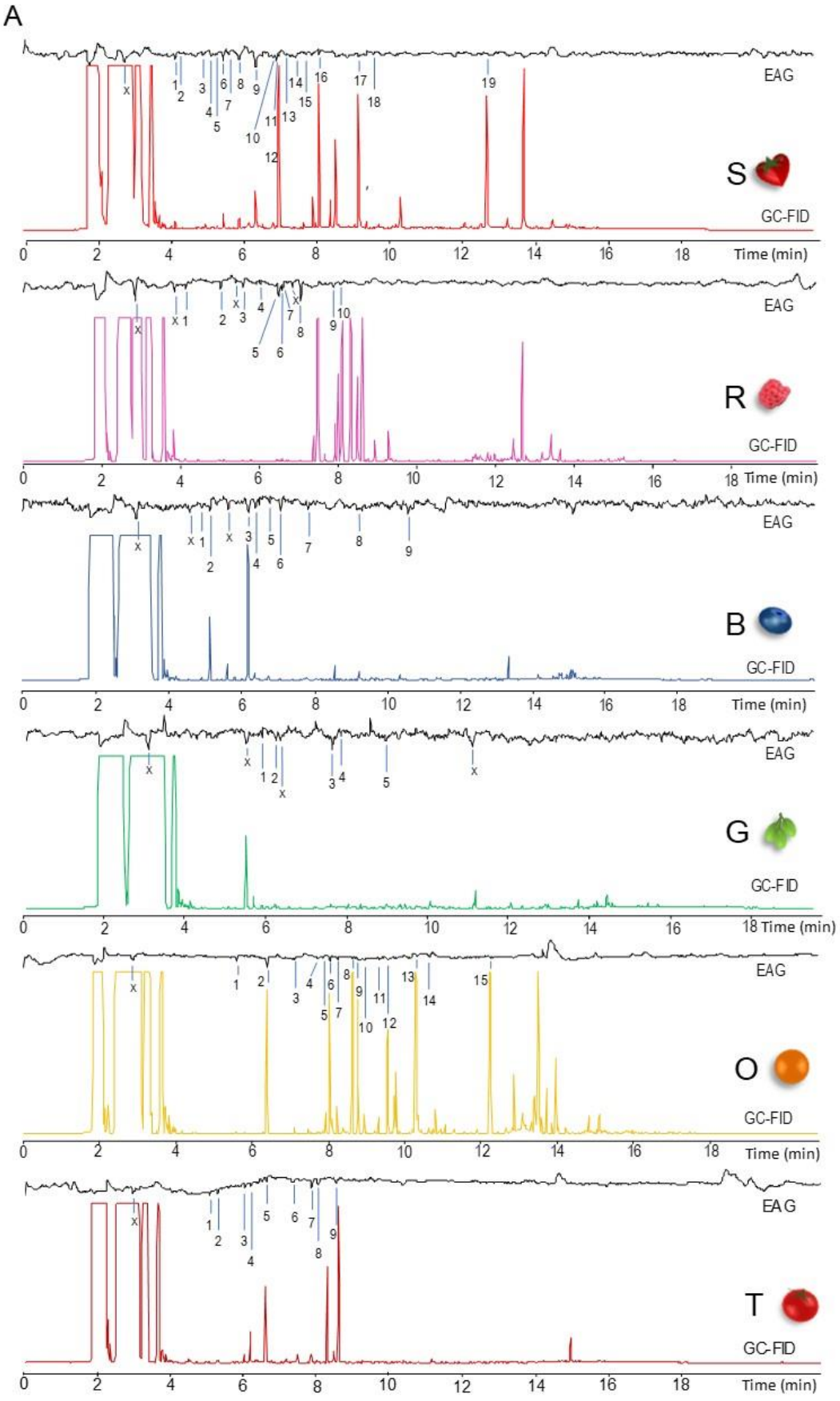
Currently, bait formulations in combination with a visually attractive trap are being created or improved. Wine (including ethanol), vinegar (or acetic acid) and sugar in a mixture have been the most attractive to Drosophilids as they released similar volatiles as fermenting yeast in Overripe fruits (Lee *et al.* 2013; Knight *et al.* 2016). Droskidrink (apple cider vinegar, red wine and sugar) in a red trap is currently deployed in Northern Italy (Pers. Comm by A. Grassi and observations) (Grassi *et al.* 2014). Another efficient trap developed, is a combination of red traps with a black stripe, and two separate baits: apple cider vinegar-ethanol, and a fermenting sugar-yeast mixture. They were the most effective traps to capture *D. suzukii* in guava orchards field trials (Lasa *et al.* 2017). Lastly, a protein bait manufactured from brewery yeast waste used in a red trap, was found far more attractive than traditional yeast/alcohol baits against *D. suzukii* (Chen *et al*. 2018). Yeast derived odours are also being developed in fruit orchards notably using the strain *H. uvarum* which was found to be preferred over other yeasts species by *D. suzukii* (Knight *et al.* 2016; Cloonan *et al.* 2018; Spitaler *et al.* 2018).

The limitation of these baits is their poor specificity as they attract many *Drosophila sp*ecies. *D. suzukii* are different from other Drosophilids by their preference for ripening fruits instead of overripe fruits (Keesey *et al.* 2015). Baits are surrounded by ripening fruits that are more attractive to the fly. Increasing the competitivity of chemical baits against fruit odours may enable to improve catching. The identification of ripening fruit volatiles inducing a strong attraction or repellency (based on the host status of the fruit type) would permit to enlarge the spectra of candidate semiochemicals for bait formulation.

4.2 *CHARACTERISATION OF ANTENNALLY ACTIVE RIPE FRUIT VOLATILES*

The aim of the first study of this chapter was to identify volatiles from ripe fruits that are detected by gravid females *D. suzukii.* Headspace volatiles from harvested fruits of strawberry, raspberry, blueberry, orange, grape and tomato plants were extracted using air entrainment (Method 2.4). Extracts were presented to gravid females in GC-EAG experiments to locate antennally active compounds (Method 2.5). Each fruit extract was injected in a GC and split, one half the GC-eluent passed through a GC-FID while the other half simultaneously fed into an airstream over a female antenna, where EAG responses were measured. Chemicals were identified from GC-peaks that elicited consistent antennal responses (EAG responses) in at least two out of three female flies (Method 2.6). A total of 55 antennally active compounds were identified from the collections of fruit headspaces with very little overlap in active chemicals among the fruits (Figure 4-1).

EAG responses were detected within the time range of the eluting solvent, indicating that there are bioactive compounds masked by the solvent peak (Figure 4-1). Another headspace collection was performed using Tenax TA polymer (Method 2.6) to identify these compounds. The extraction of chemicals was done using thermal desorption, without solvent. These extracts were not used for GC-EAG runs. Using EAG, ethyl acetate, 2-butanone and 2-hydroxy-2-butanone were identified as antennally active from strawberry and raspberry headspaces. Headspaces collected from fruits on plants and from foliage were not compared due to time constraints. Chemicals that could be tentatively identified using GC-MS were summarised in Appendix 5. Furthermore, bioactive peaks were located but the identification of the composition was not successful. The tentative identification with GC-MS revealed no match from NIST libraries, or the corresponding chemicals were not successfully co-injected or antennally active.


A
EAG
S
GC-FID
Time (min)
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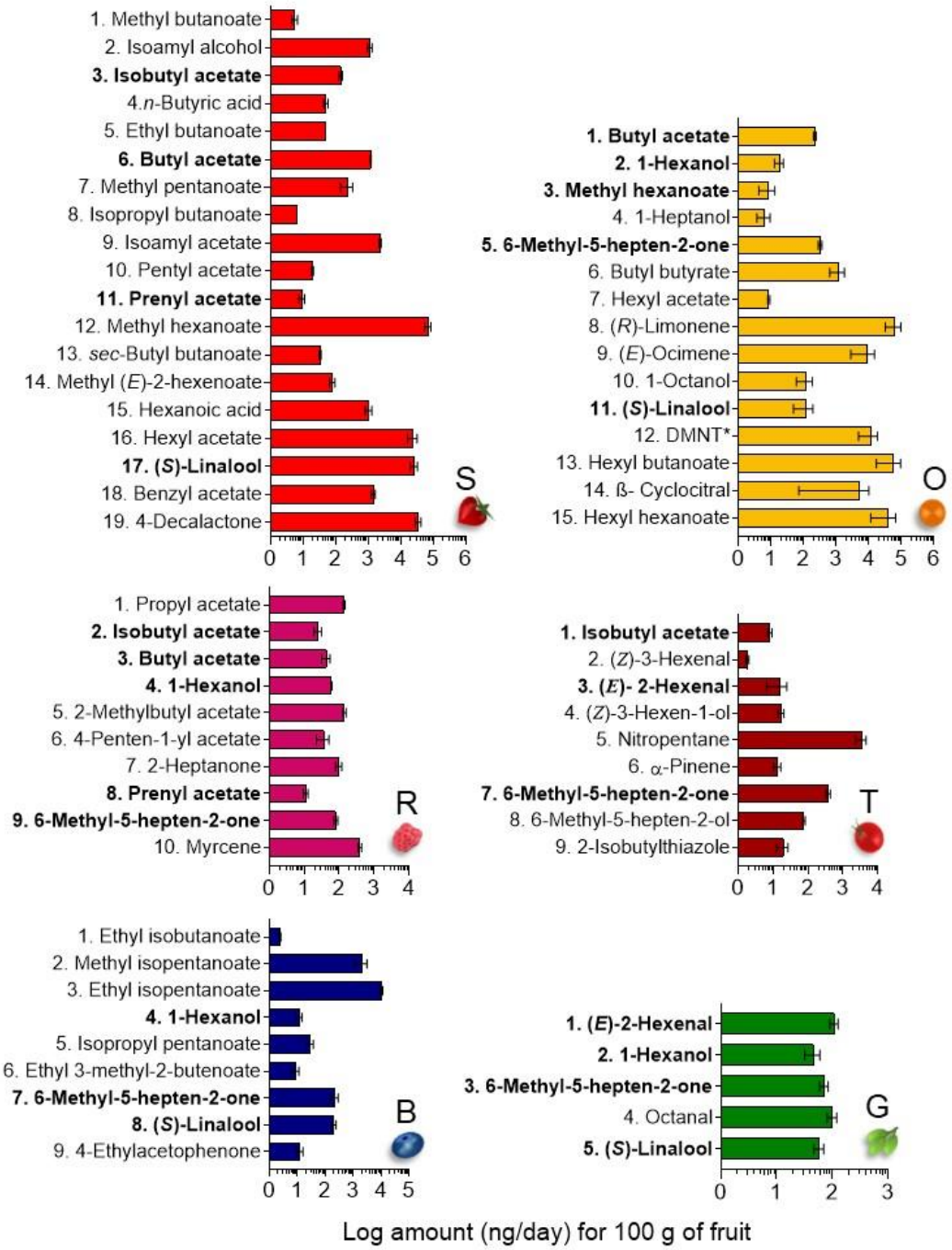


Figure 4-1 Antennally active compounds from fruit headspace extracts

A) GC- EAG traces of each fruit extract. Antenna response of gravid females *D. suzukii* is shown as the EAG trace obtained simultaneously with the sequential release of compounds (GC-FID trace). From top to bottom, the extracts were from harvested ripe whole strawberry (S), raspberry (R), blueberry (B), grape (G), orange (O) and tomato (T). "X" antenna responses which corresponding peak was unidentified. B) Mean amount ± SEM of compound released per day by 100 g of fruit (log_{10} scale). Compounds in bold are found in more than one fruit. *(E)-4,8-Dimethyl-1,3,7-nonatriene. Numbers preceding compound names correspond to FID peaks in (A).

4.2.1 55 fruit volatiles are detected by gravid females *D. suzukii*.

Compounds are released in various amounts from less than 10 ng/h to more than 50 µg/h per 100 g of fruit (Figure 4-1). Strawberry and orange released the largest amounts of compounds compared to blueberry and grape which released very low amounts. Nine compounds were found to be present in more than two fruit extracts: 6-methyl-5-hepten-2-one, (*S*)-linalool, 1-hexanol, butyl acetate, isobutyl acetate, hexyl acetate, methyl hexanoate and prenyl acetate. No active chemicals were present in all three host fruit extracts. The results therefore show that the bouquets of antennally active compounds are fruit specific. It can be concluded that *D. suzukii* does not detect chemicals that are shared by the three host fruit headspaces.

These results also show that some compounds which induced the highest antennal activity were released in the smallest amount in a fruit extract. These may be particularly involved in the recognition of host and non-host fruits. Indeed, females are highly sensitive to small doses of these chemicals. Amplitude of EAG responses were not quantitatively measured. The antennal responses were further analysed with single sensillum recordings (SSR) in order to identify which classes of ORNs are activated by these chemicals. The relevance of these compounds for host selection may be described by how their patterns of activation of ORNs matches the ones of whole fruit headspaces.

4.2.2 Chemical classes represented in fruit headspaces

Headspaces of fruits may also be similar by the types of compounds that are released. Compounds were classified by their structure and functional groups. Relative proportions of chemicals from different classes were determined. The number of chemicals and their amounts in each class were then compared (Figure 4-2). The highest representations are detailed below.

Esters represented more than 50% of the antennally active compounds identified in headspace extract from host fruits strawberry, raspberry and blueberry and approximately 40% of orange headspaces. Esters also represented more than 80% of the total amount of chemicals released by strawberry and raspberry, 38% of the amount released by blueberry and 50% of the amount released by orange. Ketones were found only in the most attractive fruit headspaces (strawberry and raspberry). The three host fruits did not commonly release any other chemical classes. The non-host grape, orange and tomato were the fruit headspaces containing the higher number and amount of alcohols and aldehydes (20-40%) which were released in traces amounts by host fruits.

It can be concluded that discrimination of hosts versus non host does not appear possible from the chemical classes released by fruits. Host fruit headspaces are mainly composed of esters, while non-hosts release alcohols and aldehydes but one host, blueberry was not as attractive as other hosts, and headspaces of the non-host orange are more similar to host headspaces compared to the two other non-hosts. It can be concluded that *D. suzukii* detect bouquets of volatiles with fruit-specific ratios of chemical classes.

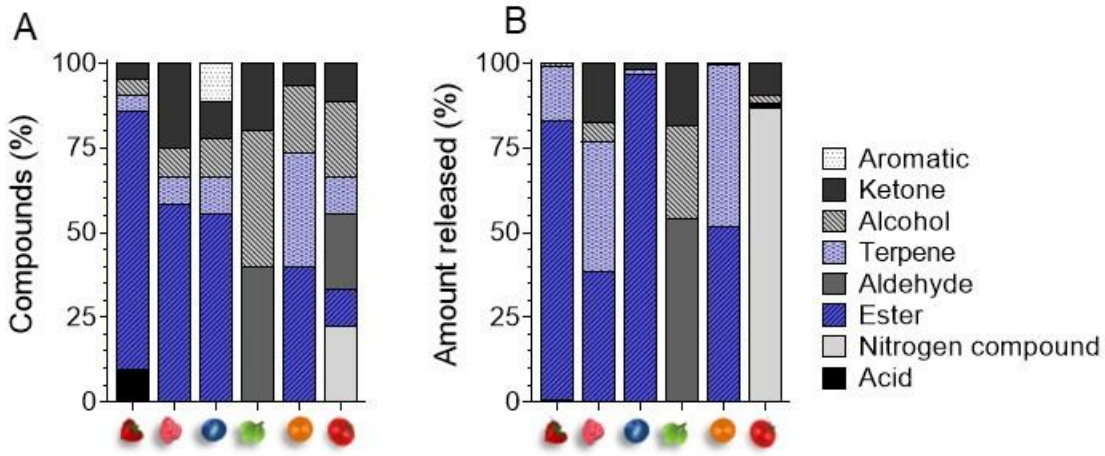


Figure 4-2 Chemical classes in fruit headspaces detected by D. suzukii
A) Relative number of compounds from different chemical classes that are detected by *D. suzukii* females from host fruit headspaces of strawberry, raspberry, blueberry and non-host fruit headspaces of grape orange and tomato (from left to right respectively). B) Relative amounts released by fruit headspaces of each chemical class (all compounds included). The terpene alcohol (*S*)-Linalool was included in terpenes only. The aromatic ketone 4-ethyl acetophenone was classified as aromatic only. Nitrogen compounds include nitropentane and 2-isobutylthiazole (Appendix 5).

4.3 DETECTION OF FRUIT VOLATILES BY SEVEN FRUIT ACTIVATED ORNS

The next step of the study aim was to determine which of the above antennally active chemicals activated the seven classes of fruit activated ORNs forming the fruitprints (Chapter 3). The 55 chemicals identified from fruit headspaces were tested for an electrophysiological response on the seven classes of ORNs which were most activated by ripe fruit headspaces (chapter 3). The response of co-localised ORNs were recorded at the same time.

4.3.1 Dose response relationships

In order to determine which doses of chemicals should be used to test the responses of the classes of ORNs to fruit volatiles, dose-response relationships of the seven fruit-activated classes of ORN with a ligand of high affinity were measured by W. van der Goes van Naters (for pb1A, ab1A, ab2A, ab3A and abXA) and the author (for ab4A and ab7A) (Figure 4-3). Dose response curves were created from responses to stimuli created with a 30 µl aliquot of diluted solution of chemical in paraffin oil from 10^{-2} to 10^{-9} µl/ml (Method 2.3).

The dose response relationships of ab1A, ab2A and pb1A to the chemical ethyl acetate were different, ab1A being strongly activated with the lowest dose; the dose inducing half the maximal response was the lowest (10^{-6} dilution). This suggest that ethyl acetate is a ligand of very high affinity for ab1A with the latter responding to very small doses compared to ab2A and pb1A. In addition, the largest does used might not have induced the maximal response from ab2A. Indeed, no plateau of saturated maximal response was obtained. This suggest that ab2A is tuned to detect differences among higher doses of ethyl acetate which saturated the responses from ab1A and pb1A.

For each of the seven ORN-ligand relationships, half the maximal response was reached with a stimulus made from dilutions between 10^{-6} to 10^{-3} µl/ml. A solution of 10^{-4} µl/ml represented an average and permitted to measure the responses from ORNs below a saturated state in most cases and to compare the affinity to fruit volatiles from each ORN class by using a single dose for all of them.

Curiously, despite similar experimental set ups, (*E*)-2-hexenal did not induce a high activation of ab4A, compared to other ORN-ligand dose responses (Figure 4-3) and compared to reported measurement on *D. melanogaster* in literature (De Bruyne *et al.* 2001).

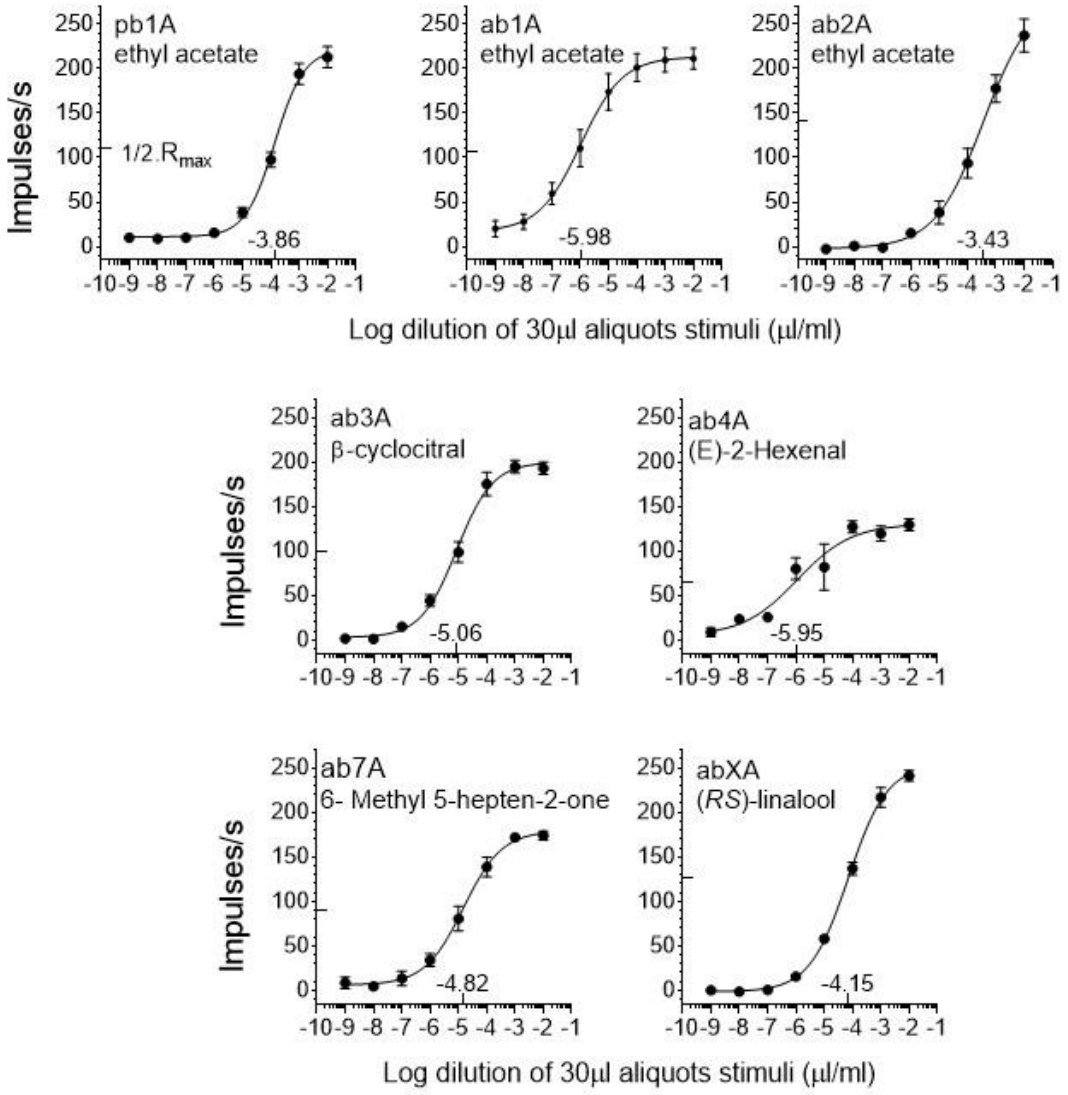


Figure 4-3 Dose response relationships for seven fruit activated classes of ORNs
A 4-parameter sigmoid Hill curve was fitted: $Y=R_{min} + (R_{max}-R_{min}) / (1+(10^{(LogDEC50-X)}) *n_H)$ with R the response (impulses/s), DEC50 the dose which gives half the maximal response (R_{max}), and n_H the Hill coefficient (or slope). DEC50 is annotated on each graph, along with ½ R_{max}. N= 6. Data for pb1A, ab1A, ab2A, ab3A and abXA were collected by W. van der Goes van Naters. 30 µl of each dilution (µl/ml) in paraffin oil was used on filter paper. The chemicals were identified from ripe fruit headspace extract and were ligands of high affinity used for the discrimination of the classes of ORNs.

4.3.2 Affinity for fruit volatiles

The seven fruit-activated ORN classes, pb1A, ab1A, ab2A, ab3A, ab4A, ab7A and abXA (Chapter 3) and co-localised ORNs were tested for electrophysiological response with each of the 55 compounds identified from fruit headspace extracts using 30 µl of a 10^{-4} µl/ml dilution on filter paper as a stimulus.

4.3.2.1 Host fruit headspaces

In the three host fruit headspaces of strawberry, raspberry and blueberry more than 50% of the antennally active volatile activated ab3A with > 50 impulses/s increase from the spontaneous activity. The second most activated ORNs were ab1A and pb1A, with 38-50% and 22-57% of host fruit volatiles induced increased activities > 50 impulses/s. By contrast, less than 33% of antennally active compounds identified from the non-host headspaces of grape, orange and tomato induced an increase above 50 impulses/s from these three classes of ORNs (Figure 4-4).

Ethyl acetate and acetoin (3 hydroxy-2-butanone) appear as most active ligands from raspberry headspace for ab1A and pb1A. However, no quantification could be made from the headspaces, it is thus unclear how much of these are released in comparison to the other volatiles. Ethyl acetate was identified from additional headspace collections of both host fruits (Appendix 5). It induced the highest activity from ab2A among all 55 compounds tested. Methyl acetate was later found, but was not tested for electrophysiological response, and may be an additional ligand for ab2A (pers. communication by W. van der Goes van Naters).

75% of the antennally active compounds identified from the host fruit blueberry headspaces strongly activated (>100 impulses/s increase) the host activated ORNs ab1A, ab2A, ab3A and pb1A. Ethyl isobutanoate was released in traces amounts (~2 ng/day, 100 g of fruits) and is a ligand of high affinity for these four host- activated ORNs. With the largest amounts released (>1 µg/day, 100 g of fruits) ethyl isopentanoate and methyl isopentanoate were among the ligands of highest affinity for ab3A (>150 impulses/s increase). Despite being present in one of the lowest amounts (<10 µg/day, 100 g of fruits) ethyl 3-methyl-2-butanoate induced strong responses from ab3A and ab7A (>150 impulses/s increase) (Figure 4-4).

Lastly, only a few ligands were identified for pb1A and for ab2A. Their affinity for these two ORNs does not reflect the strong activation of these ORNs by the headspaces of the fruits and they were not quantified from the extracts. Additional compounds may have been overlooked which may be ligands for these (Appendix 5).

4.3.2.2 Non-host headspaces

Non-host fruit headspaces of grape, orange and tomato were characterised by their fruit specific ORN activation of ab4A, ab7A and abXA, the latter being also activated by the attractive strawberry headspaces. From these headspaces, a minority of ligands induced responses of host activated ORNs and were found in the smallest amounts.

(*E*)-2 hexenal and 6-methyl-5-hepten-2-one activated ab4A and ab7A and were the most abundant and active volatile identified from grape headspaces. Some GC-FID peaks induced large EAG responses, but their composition could not be identified including one of a very large amplitude (Figure 4-1). This unknown component may be a ligand for ab4A, as no other ORN were activated by these grape headspaces.

Four compounds with high affinity to ab7A were identified from fruit headspaces: 6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol, prenyl acetate and ethyl-3-methyl-2-butanoate. The 6-methyl-5-hepten-2-one was present in the non-host tomato and orange headspaces in the largest amounts (approximately 300 ng/day for 100 g of fruit) compared to other fruits, and not found in strawberry headspace. In addition, tomato released 6-methyl-5-hepten-2-ol (approximately 77 ng/day, 100 g of fruits) prenyl acetate (<10 ng/day, 100 g of fruits) and ethyl 3-methyl-2-butanoate (<10 ng/day, 100 g of fruits). The two latter were also found in blueberry and raspberry headspaces which did not activate ab7A (Chapter 3). It was concluded that ab7A activation by tomato headspace appear to be explained by a larger quantity of 6-methyl-5-hepten-2-one released, compared to other fruits.

The activation of abXA by orange headspace appear more specific compared to the activation by strawberry headspace. (*S*)-linalool, (*E*)-4,8-Dimethyl-1,3,7-nonatriene (DMNT) and (*E*)-ocimene induced strong responses (>100 impulses/s) and are released in large amounts by oranges compared to other compounds (Approximately 100 µg/day, 100 g of fruits). In strawberry headspaces, only (*S*)-linalool was identified.

4.3.3 Conclusion on the identification of antennally active fruit volatiles

It can be concluded that fruits release specific bioactive blends of chemicals with very little overlap and no chemicals enable *D. suzukii* to distinguish the three hosts from the three non-hosts. These compounds activated specific and overlapping classes of ORNs and no chemical simultaneously activated all ORNs forming one fruitprint. In addition, some chemicals appear most relevant compared to others, as they activated many classes of ORNs with high intensity despite being released in the lowest amounts. Host fruits have in common the activation of ab1A and ab3A by many ligands, most are esters. These two classes of ORNs appear the most sensitive to ripening fruit volatiles.

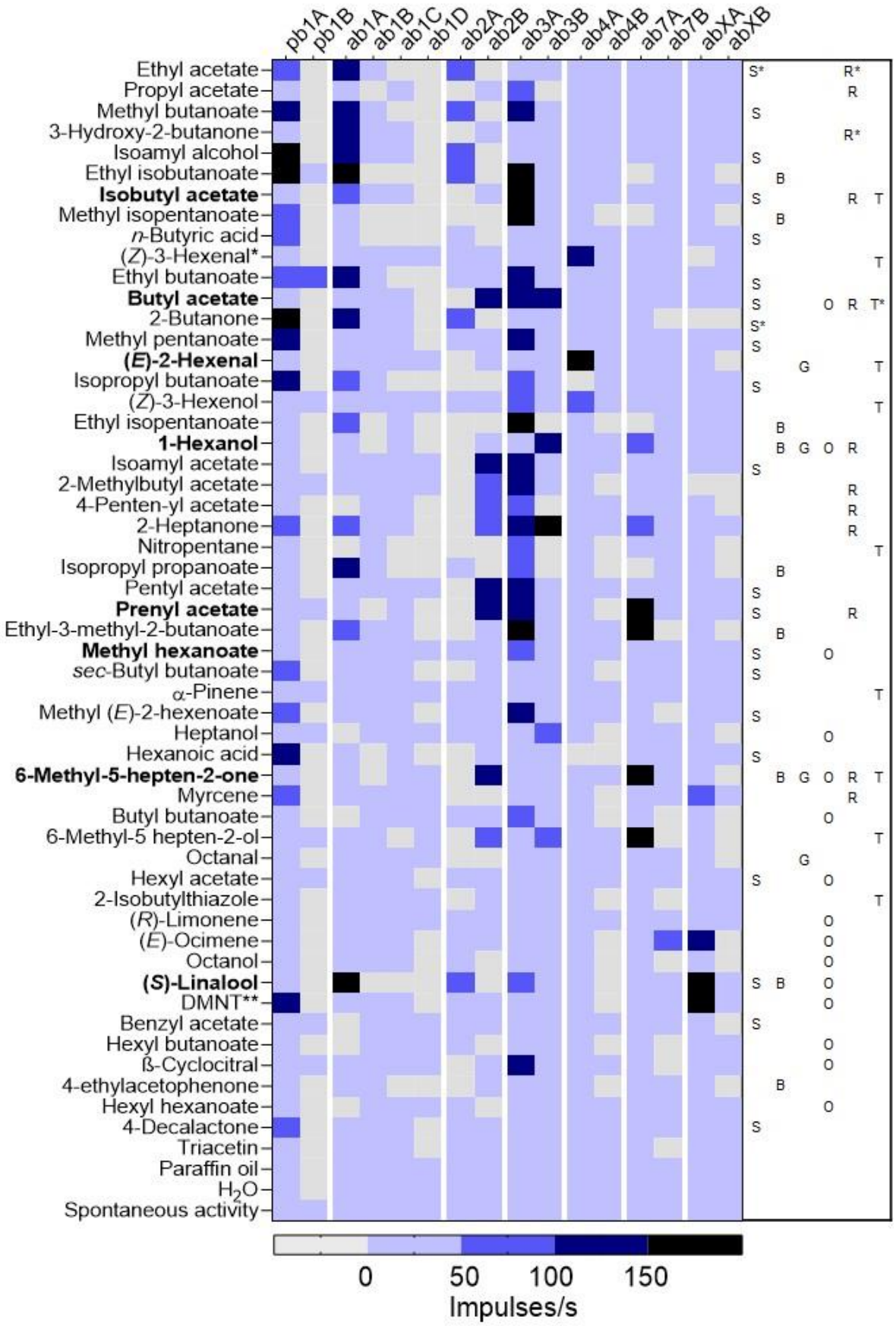

pb1A
pb1B
ab1A
ab1B
ab1C
ab1D
ab2A
ab2B
ab3A
ab3B
ab4A
ab4B
ab7A
ab7B
abXA
abXB
Ethyl acetate
Propyl acetate
Methyl butanoate
3-Hydroxy-2-butanone
Isoamyl alcohol
Ethyl isobutanoate
Isobutyl acetate
Methyl isopentanoate
n-Butyric acid
(Z)-3-Hexenal*
Ethyl butanoate
Butyl acetate
2-Butanone
Methyl pentanoate
(E)-2-Hexenal
Isopropyl butanoate
(Z)-3-Hexenol
Ethyl isopentanoate
1-Hexanol
Isoamyl acetate
2-Methylbutyl acetate
4-Penten-yl acetate
2-Heptanone
Nitropentane
Isopropyl propanoate
Pentyl acetate
Prenyl acetate
Ethyl-3-methyl-2-butanoate
Methyl hexanoate
sec-Butyl butanoate
α-Pinene
Methyl (E)-2-hexenoate
Heptanol
Hexanoic acid
6-Methyl-5-hepten-2-one
Myrcene
Butyl butanoate
6-Methyl-5 hepten-2-ol
Octanal
Hexyl acetate
2-Isobutylthiazole
(R)-Limonene
(E)-Ocimene
Octanol
(S)-Linalool
DMNT**
Benzyl acetate
Hexyl butanoate
ß-Cyclocitral
4-ethylacetophenone
Hexyl hexanoate
4-Decalactone
Triacetin
Paraffin oil
H2O
Spontaneous activity
0
50
100
150
Impulses/s

Figure 4-4 Olfactory receptor neurons (ORNs) responses to ripe fruit volatiles in D. suzukii

Mean impulse rate during a 0.5 s stimulus with 30 µl of a 0.01% solution in paraffin oil or H_2O. The higher the increase in activity compared to the spontaneous activity, the darker the colour. *(*Z*)-3-Hexenal was present in a 50:50 ratio with triacetin, a stabilizing agent. Only the functional basiconic types housing a fruit activated class of ORN (chapter 3) were recorded from on gravid females *D. suzukii*. Volatiles were characterised from fruit headspaces of strawberry (S), raspberry (R), blueberry (B), grape (G), orange (O) and tomato (T). Compounds in bold are common to two or more fruits. Compounds are ranked from the highest to the lowest volatility.,

4.4 BEHAVIOURAL RESPONSES TO THE ACTIVATION OF DEDICATED SUBSETS OF ORNS

How are behavioural responses to fruit odours mediated by the peripheral olfactory system? The next study of this chapter aimed to determine which of the fruit activated ORNs mediate behavioural responses. It may be hypothesised that: 1) not one ORN classes is sufficient to mediate attraction but a combination of multiple; 2) multiple combinations of ORNs mediates attraction to different fruit types instead of a single combination commonly activated by all host fruits (Figure 1-4). It can also be hypothesised that it may be possible to mimic or surpass a fruit headspace attractiveness using a blend of chemicals (or a single chemical) which gives the same or a stronger fruitprint. This would permit to design novel attractive mixture to use in baits, which would be competitive with fruits in orchards.

These hypotheses were addressed with behavioural experiments using the results collected above. The first objective was to induce attraction by re-creating the fruitprint of strawberry. The second objective was to activate a different combination of ORNs, using isoamyl acetate to mediate attraction. The last objective was to decrease the attraction to fruit by activating non-host ORNs.

4.4.1 The simultaneous activation of pb1A and ab1A mediate attraction

4.4.1.1 Tentative imitation of strawberry headspaces via activation of the same ORNs

The first aim was to recreate the activation pattern of strawberry headspace (fruitprint) by activation of ab1A, ab2A, ab3A, pb1A and abXA. Four ligands were simultaneously presented to gravid females in a 4-choices assay and in a wind tunnel assay.

Methyl propionate, ethyl acetate, (S)-Linalool and β-cyclocitral were chosen because they specifically activate at least one of the five ORNs (Figure 4-3). Methyl propionate was not identified from fruit headspaces but was chosen as a known ligand for ab2A. Four capillaries

were housed together to form a bait in a 4-choice cage assay. Capillaries each contained approximately 30 µl of a 1% (or 10^{-4} g/ml) dilution of one chemical in paraffin oil. The release rates of the four compounds during the behavioural assay were determined with GC to verify which doses were presented to gravid females. Headspaces were collected for one hour, starting the first hour after the preparation of the capillaries (hence start of the behavioural test) and starting three hours after the preparation (hence three hours after the start of the behavioural assay) (Figure 4-5). The experiment showed that the chemicals were released during the whole duration of the assay in amounts that can be detected by females: >150 ng/h. Lower amounts induced antennal responses in fruit extracts (Figure 4-2). The experiment was repeated in two different laboratories, at Rothamsted Research and at Cardiff University, using two populations of flies (Method 1.1). The results of both experiments were statistically tested using a GLM on the number of flies (Appendix 6).

After five hours, significantly more females were collected from the bait with the odours than from the controls suggesting that the activation of ORNs by these four compounds was attractive. Both repeats showed similar results indicating that the attraction of the four compounds was consistent (Figure 4-5, Appendix 6). The bait was as high attractive as strawberry headspaces, with approximately 50% more females being collected odour baits compared to controls (Chapter 3). It was concluded that the four capillaries imitated strawberry headspaces via activation of the five classes of ORNs.

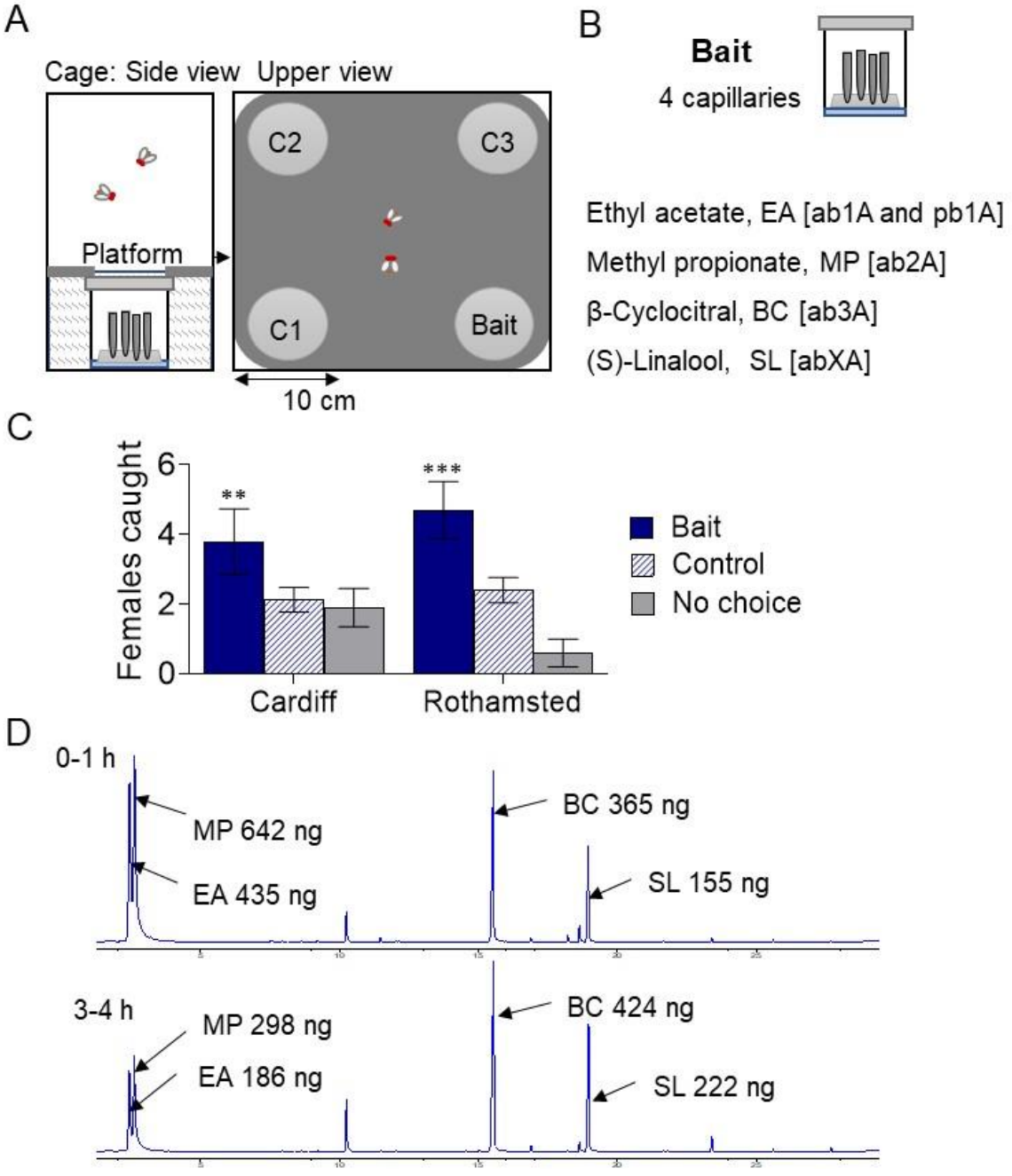


Figure 4-5 Behavioural attraction via activation of the same ORNs as the strawberry headspaces

A) Scheme of 4-choice cage assay. B) Capillaries contained each a 10^{-2} µl/ml (1%) solution of ethyl acetate (EA), methyl propionate (MP) β-cyclocitral (BC) and (S)-linalool (SL). ORNs expected to be activated in brackets. C) Mean (± SEM) number of females collected from bait, control and cage (no choice) in assays done in two separate experiments in Cardiff and Rothamsted. The number of females on the three controls were not different and were represented as an average of the three. Numbers were statistically compared with a GLM Poisson Distribution: ***, P<0.001, ** P<0.01 (Appendix 6). C) GC-FID peaks from the four compounds from the bait analysed following 1 h headspace collection at the start (top) and after 3 h (bottom).

In a separate experiment, a blend of the four chemicals was tested in a wind tunnel. The objective of this procedure was to test a mixture in which compounds would be released in low concentrations to activate only the most sensitive classes of ORNs. A first attempt consisted of a mixture which released doses that induce approximately half the maximal response of each class of ORNs (Figure 4-3). This so called EC50 blend contains 48% ethyl acetate, 19% methyl propionate, 3% β-cyclocitral and 30% (S)- linalool. Ethyl acetate and (*S*)-linalool were also tested as single compounds, using a 1% solution. Chemicals were loaded on filter paper using a solution in paraffin oil. β-cyclocitral and methyl propionate and other concentrations could not be tested due to time constraints. Females were significantly more attracted to a bait containing one of the chemicals or the mixture, compared to a control bait. Indeed, once upwind females evenly distributed on both baits except when presented with ethyl acetate, on which bait more females were collected (Figure 4-6, Appendix 6).

Results suggest that the simultaneous activation of the five ORNs as with strawberry headspaces induced attraction of gravid females *D. suzukii*. Strawberry headspaces were still more attractive compared to the blend and compounds in the wind tunnel (approximate PI of 0.70, Chapter 3) therefore, the blend was a poor imitation of the fruit headspace in the wind tunnel. The expected simultaneous activation of ab1A, ab2A and ab3A by ethyl acetate and of abXA, ab1A and ab2A by (S)-linalool have induced a similar attraction than the blend.

From these two behavioural experiments (wind tunnel and 4-choice cage assay), it is unclear whether the pattern of activation of ORNs was similar to the strawberry fruitprint as expected. How many classes of ORNs were activated? With which intensity?

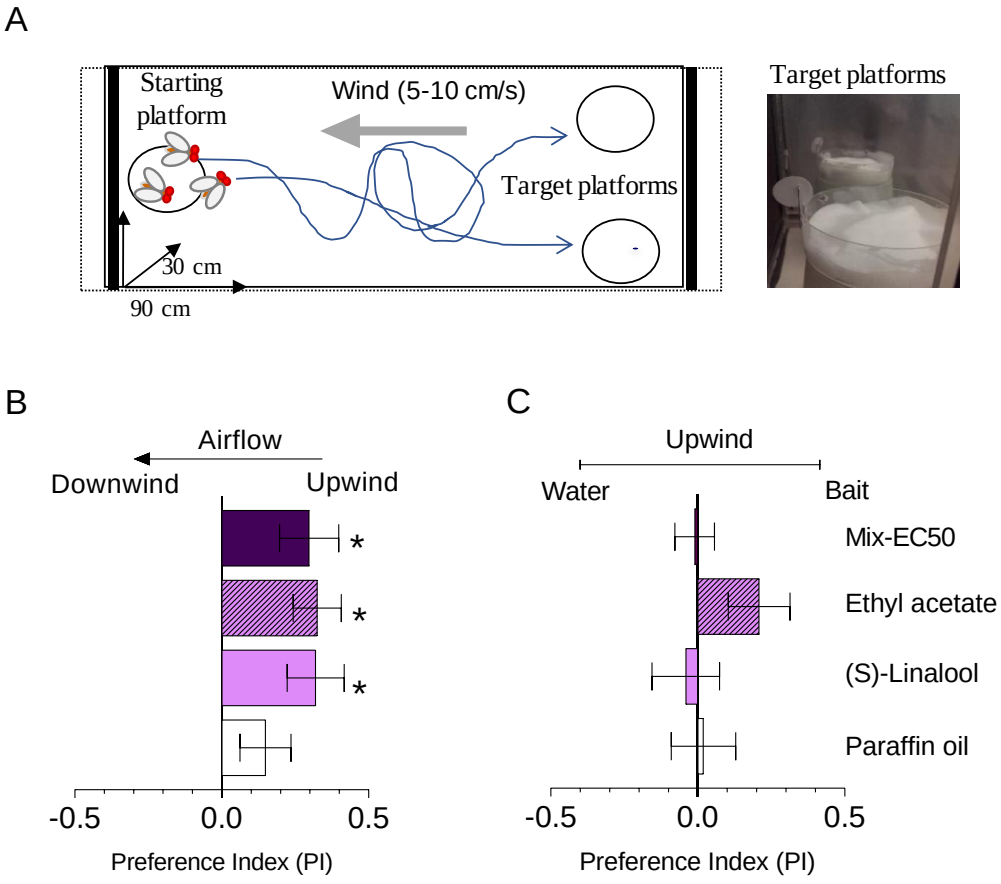


Figure 4-6. Behavioural response to fruit volatiles activating specific ORNs.
A) Wind tunnel assay with compounds loaded on filter paper, using paraffin oil as solvent (10 µl). Mix-EC50 is composed of a blend of 48% ethyl acetate, 19% methyl propionate, 3% β-cyclocitral and 30% (S)- linalool, corresponding to the dose of chemicals which induced half the maximal ORN response in dose-response experiments. B&C) Mean (± SEM) preference index showing the attraction to upwind baits (B) and the distribution between bait and control (C). Baits are Mix-EC50 (dark purple), ethyl acetate (striped purple), (S)-linalool (light purple), paraffin oil (control, white). All were simultaneously presented with a control bait. N=6-9 replicates with 10-20 females. Sample-t-test: *, P<0.05.

4.4.1.2 Attraction by activation of ab1A and pb1A

To decipher which combinations induced an attraction of females, the results in the 4-choice cage assay were investigated and each component was tested with additional experiments. Each of the four compounds was tested as a single bait in the same quantity and concentration as when tested together (1 capillary with one 1% dose and 3 capillaries with paraffin oil) , in the 4-choice cage assay.

Significantly more females were collected on the bait containing methyl propionate compared to the controls, containing only paraffin oil (Figure 4-7). Other compounds tested alone were not more attractive than controls (Appendix 6). Ethyl acetate, which was attractive in the wind tunnel (Figure 4-7) was not more attractive than controls in the 4-choice cage assay, suggesting that the detection of the bait was different in the two assays.

In order to identify why attraction was induced by methyl propionate but not by ethyl acetate in the 4-choice cage assay, headspaces above these two baits were tested for electrophysiological responses. The bait containing methyl propionate activated both pb1A and ab1A with > 50 impulses/s, and ethyl acetate activated only ab1A with > 50 impulses/s (Figure 4-7). It can be concluded that the simultaneous activation of pb1A and ab1A by methyl propionate induced attraction. Furthermore, the activation of ab1A alone (by ethyl acetate) was not sufficient to induce attraction. In addition, the activation of ab2A and ab3A were not necessary to induce attraction of gravid female *D. suzukii*.

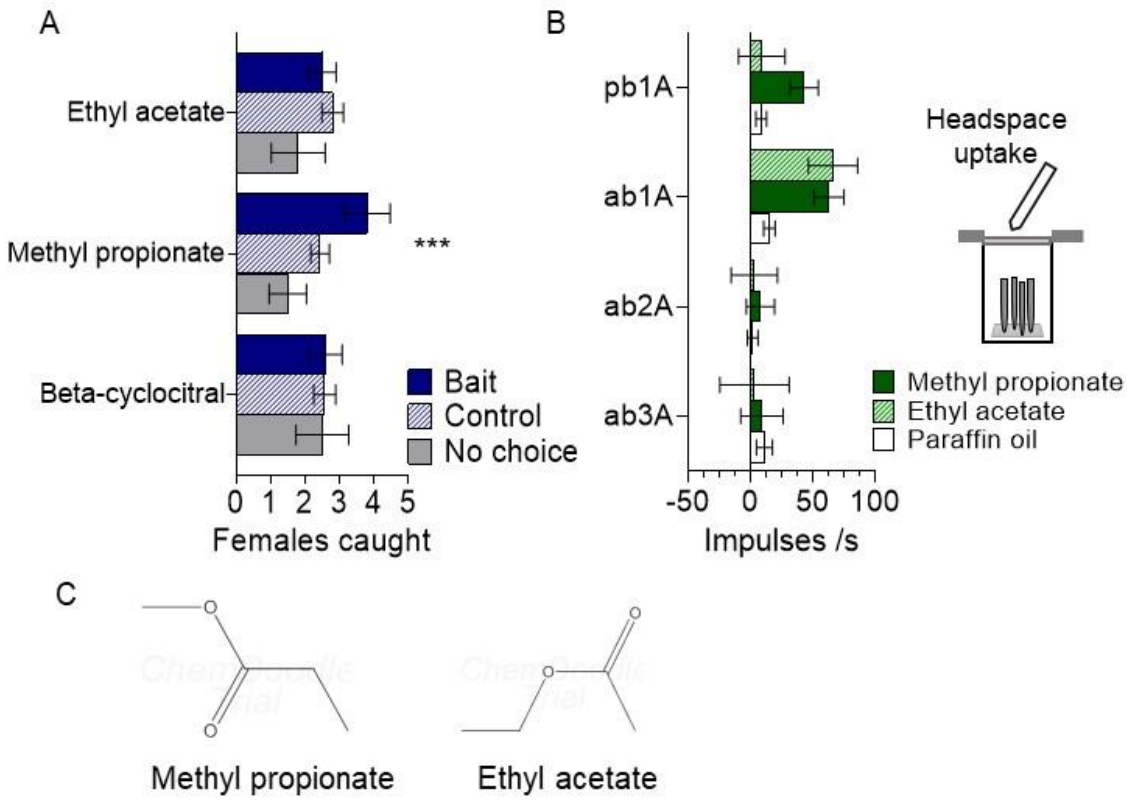


Figure 4-7 Simultaneous activation of ab1A and pb1A ORNs induced an attraction of gravid females.

A) Mean number (± SEM) of females collected in a 4-choice cage assay on bait, controls and cage (no Choice). The numbers of females on the three controls were not different so an average was represented. N=10. The numbers on the bait were significantly different from control and no Choice GLM with Poisson Distribution: ***, P<0.001 (Appendix, Table 10-10). N=10. B) Mean (± SEM) number of impulses/s during a 0.5s

stimulus with headspaces collected above the bait. N= 4-9 replicates with 10-20 females. C) 2D -conformation of ethyl acetate and methyl propionate females.

4.4.1.3 Behavioural and antennal response to linalool

The compound Linalool was used as a racemic mixture for the characterisation of the ORNs and was the ligand with highest affinity for abXA. However, only the (S) enantiomer was identified in fruit extracts from host and non-host (Figure 4-1). It induced attraction when tested alone in the wind tunnel but not in the 4-choice cage assay (Appendix 6). The above experiment was modified to test the enantiomer (R)- Linalool in addition to the three other chemicals. Three baits were tested in the 4-choice cage assay, one contained a racemic mixture (Bait *RS*), one contained only (S)- linalool (Bait-1/2S) at the same dose as in the racemic mixture and one contained twice the amount of (S)-linalool (Bait-S) (Figure 4-8).

Significantly more females were collected from the baits without (R)-linalool (Bait-S and Bait -1/2S) than from controls. The number of females which had not selected a bait (No choice) was the highest when the bait contained (R)-linalool. (S)-linalool when tested alone, did not induce a behavioural response from gravid females, as they randomly distributed on baits and in the cage. This experiment suggested that both a reduction of (S)-linalool and an addition of (R)-linalool in the Bait-*RS* contributed to a loss of attraction, but that (S)-linalool was not the only one eliciting attraction.

4.4.1.3.1 The non-attractive bait activated abXA

The next step was to determine how the two baits were detected, to identify the classes of ORNs contributing to the loss of attraction when (R)-linalool was added. The headspaces of the baits were collected above the bait and used as a stimulus in SSR on the seven fruit-activated ORNs. The recordings on ab4A were not successful. Despite an additional dilution of the headspace in the air stream, this technique permitted to test as a stimulus, the headspaces as they were released in the behavioural assay.

The major difference noted was that Bait-*RS* activated abXA with higher impulse rate than Bait-S, with approximately 40 impulses/s. In addition, Bait-S activated only pb1A and ab1A with 50-100 impulses/s and induced a significant attraction of gravid females (Figure 4-8).

It can be concluded that the simultaneous activation of ab1A and pb1A induced attraction in two separate experiments (Figure 4-5, Figure 4-7) and that an activation of 40-50 impulses/s may be sufficient to induce a behavioural response. This experiment also shows that the activation of ab2A and ab3A is not necessary to induce attraction of gravid females *D. suzukii* (Figure 4-6). The chemicals were not released in sufficient amounts to induce the expected activation of ab2A and ab3A. This study also suggests that abXA may have induced a decrease in attractiveness.

4.4.1.3.2 Enantiospecific pattern of activation

To determine if other classes of ORNs differed in their response to the two enantiomers hereby having enantio-specificity, (S)-linalool, (*RS*)- linalool were tested as single stimulus (Figure 4-8). Most notable differences are that the ab7A and the ab2B are activated with > 50 impulses/s by the racemic mixture but not by the (S)-linalool which mostly activated ab2A and ab3A and ab3B. It can be concluded that an activation of ab7A and ab2B is associated with non-attraction and perhaps repellence.

Both enantiomers were then tested in single form stimulus with a lower dose (0.01% dilution) as were the compounds identified from fruit headspaces (Figure 4-2). (S)-linalool found in ripening fruits induced a strong activation (> 150 impulses/s) of ab1A and abXA followed by ab3A and ab2A (approximately 100 impulses/s). (R)-linalool, not found in the fruits used in this study, activated only pb1A with > 50 impulses/s. It can be concluded that both enantiomers are detected by different combinations of ORNs: the classes ab1A, ab2A, ab3A and abXA appear enantio-specific for linalool.

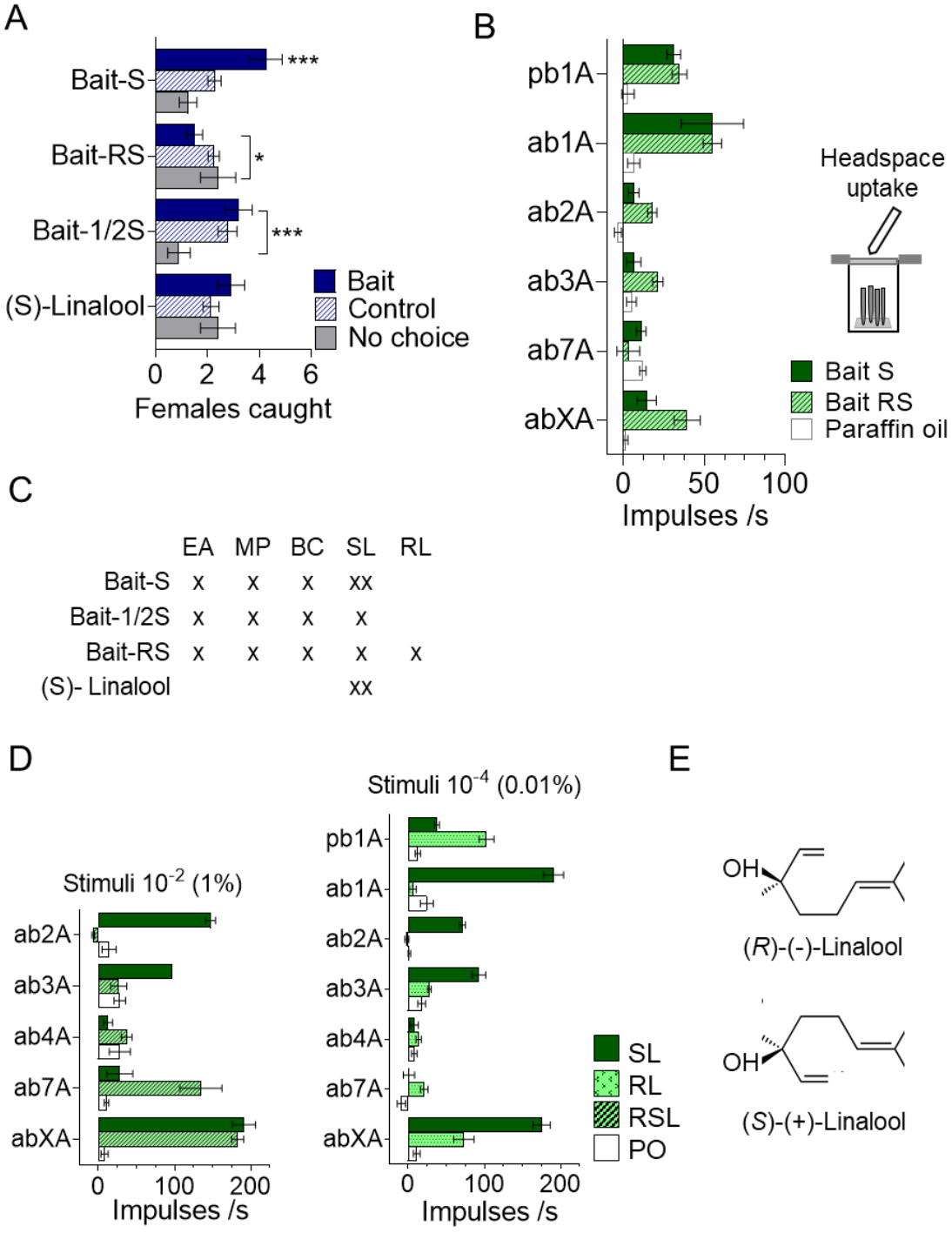


Figure 4-8 Differential ORN responses to the enantiomers of Linalool.

A) Mean number (± SEM) of females collected in a 4-choice cage assay, on bait, controls and cage (no Choice). The number of females on the three controls were not different so an average was represented. N=10. The numbers on baits were significantly different from control and/or no Choice. GLM with Poisson Distribution: ***, P<0.001, * P<0.05 (Appendix 6). Bait-S is a pool of the data shown Figure 4-5. B) ORNs response rates (mean ± SEM) to the headspaces above the baits containing ethyl acetate (EA), methyl propionate (MP), β-cyclocitral (BC) and (S)-linalool (SL) or (*RS*)-linalool (*RS*). N=1-15).

C) Composition of the baits used in A & B. 'x' one dose of chemical at 1%. The racemic mixture contained one dose of each enantiomer. Bait 1/2S contained one dose, hence as much as in the racemic mixture. D) Response rate (mean ± SEM) of ORNs to a racemic (50:50) mixture of (*RS*) -Linalool (stripped blue), (S)-linalool (dark blue) and (R)-linalool (dots, light blue) during a 0.5s stimuli with 1% (left) and 0.01% (right) dose on filter paper. PO (white) are the responses to paraffin oil. N= 1-6. Recordings for (S)-linalool at 0.01% and for (*RS*)-linalool at 1% were taken from the used datasets for Figure 4-4, and Table 10-15 respectively. E) 2D conformation of (S)- and (R)-linalool.

4.4.2 Isoamyl acetate elicited attraction of gravid females

The second objective of this study was to activate a different set of ORNs, using isoamyl acetate to mediate attraction. Isoamyl acetate was chosen as a case study because it was reported to be attractive and of importance for *D. suzukii* by Revadi *et al.* (2015). In addition, isoamyl acetate was identified as the most active compound from strawberry headspaces (see above) despite not being released in the largest amount (Table 4-2).

A wind tunnel with rubber septa was used to test the behavioural response of gravid female *D. suzukii* to isoamyl acetate, as described in Revadi *et al.* (2015). The same dose as used in their study was also used before to continue with further testing. Two tests were performed using 10 µl of a 1% solution in hexane, of isoamyl acetate from two stocks.

The solution from one of the stocks was significantly attractive while the other was not (Figure 4-9, Appendix 6). Using GC-MS and NMR techniques (Method 2.3.3) the purity of the two solution was verified by D. Withall (Rothamsted Research). The stock which was not attractive was a blend containing approximately 67% isoamyl acetate, 29% 2-methyl-butyl acetate and 4% 4-hydroxy-4-methyl-2-pentanone (so called diacetone alcohol), while the attractive stock contained isoamyl acetate with over 98% purity (NMR outcome not shown).

The experiment shows that the attractiveness of isoamyl acetate was lost when the compound was in a mixture with the two other compounds. Isoamyl acetate was significantly more attractive than the control, and the attracted females on the bait containing the odour source outnumbered the females collected on the control (the preference was calculated for females reaching the upwind platforms, see method 2.8). On the contrary, the few females attracted by the blend appeared to have avoided the source of the odour as more of them were collected on the control bait. However, additional replicates are needed (Figure 4-8). The decrease attractiveness of the blend compared to isoamyl acetate may be explained by i) a lower amount of isoamyl acetate is presented to the females; ii) 2-methyl butyl acetate and /or diacetone alcohol induced a decrease of attraction.

In the present study, 30 µl of a 0.01% dilution of isoamyl acetate commonly activated only ab2B and ab3A with > 100 impulses/s, and ab7A with approximately 50 impulses/s. Surprisingly, ab1A was not activated at this dose while being the most sensitive at a higher dose. The ab3A and ab2B neurons were therefore the most responsive to isoamyl acetate, but because the activation pattern in the wind tunnel was not determined, it is unclear whether they were sufficient to mediate a behavioural response. The class pb1A however, which was associated with attraction in the section above, was not activated in both doses tested, suggesting that its activation was not necessary.

2-methyl butyl acetate was identified from raspberry headspace extract and its activation pattern (with a 0.01% solution) was like the one of isoamyl acetate (Figure 4-9). It is unclear whether the two compounds have similar affinities in the doses released in the wind tunnel, and if the mixture of the two have agonist, neutral, additive or synergistic effects. The activation pattern of diacetone alcohol was not determined. Additional tests are required to determine the cause of a reduced attraction.

Rubber septa is an adequate support to slow release fruit volatiles in a laboratory behavioural assay lasting 5 h, as demonstrated with isoamyl acetate. Additional tests are required to test compounds of various volatility and their mixture.

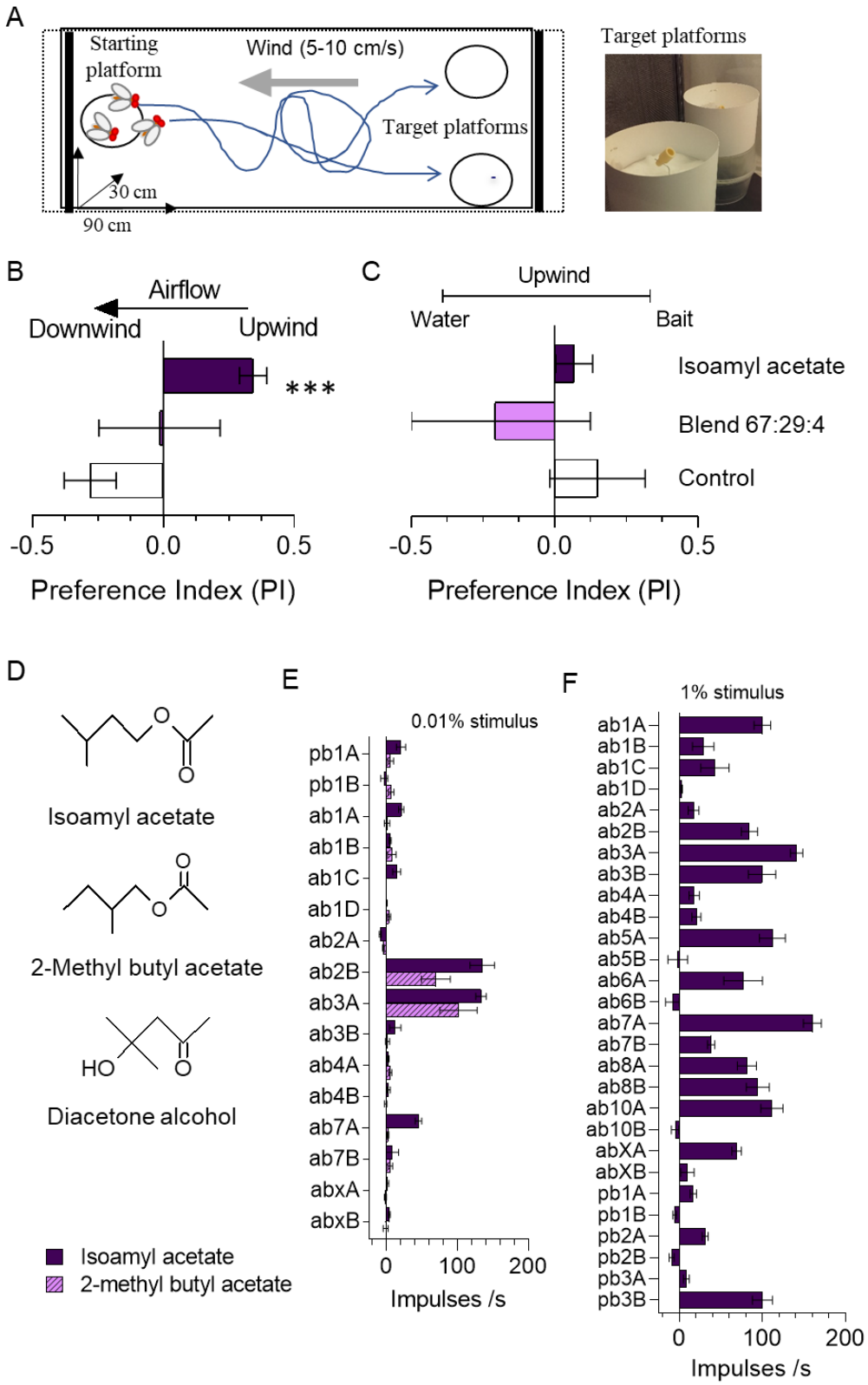


Figure 4-9 Isoamyl acetate induced attraction via activation of at least ab3A and ab2B

A) Wind tunnel assay with rubber septa. B-C) Mean (± SEM) preference index showing the attraction to upwind baits (B) and the distribution between bait and control (C). Baits

were isoamyl acetate (dark purple), a mixture of 67% isoamyl acetate, 29% 2-methyl-butyl acetate and 4% diacetone alcohol (Blend 67:29:4, light purple), Hexane (control, white). All were presented next to a control. N= 4-8. Sample t-test: ***, P < 0.001. D. 2-D conformation of the three volatiles. E&F) Mean (± SEM) response rate of ORNs during a 0.5 s stimulus with 30 µl of: E) 0.01% solution of isoamyl acetate (dark purple) and 2-methyl-butyl acetate (light purple); F) 1% solution of isoamyl acetate. Data for E&F originated from appendix 2 and figure 4.4.

4.4.3 Candidate masking or repellent cues

The aim of the next series of experiments was to tentatively identify an olfactory circuitry by which masking or repellent odours are processed. The type ab7A was identified as a class of ORN that is activated by non-host fruits and not by the host fruits tested. Tomato is an unattractive fruit whose headspace mainly activated ab7A (Chapter 3). In the first part of this study, ligands for ab7A were identified from tomato headspaces, the most abundant being 6-methyl-5-hepten-2-one (Figure 4-1). 6-methyl-5-hepten-2-one is also a ligand for ab2B, yet this ORN class was not activated by headspaces (Chapter 3). In addition, tomato headspace contained 6-methyl-5-hepten-2-ol and prenyl acetate, both of which also activated ab7A with high intensity (Figure 4-4).

4.4.3.1 Tomato headspace masks raspberry headspaces

In order to determine whether activating ab7A would mediate non attraction and perhaps mask the attractiveness of host fruits, an experiment was performed in which tomato headspaces and raspberry headspaces were simultaneously presented to gravid female *D. suzukii*. The two fruit types were housed together in a single bait, in which different amounts of raspberry were given with one tomato. The bait was given as a choice along with three control baits (water).

A significant decrease in the number of females was observed on the bait housing the two fruits compared to the bait with only the host fruit raspberry (Figure 4-10). The results also showed that attractiveness was restored when more attractive headspaces were offered (via increase in the number of raspberries). It can be concluded that tomato headspace decreased the attractiveness of raspberry headspaces. This result led to the hypothesis by which the activation of ab7A reduces the attractiveness of a host fruit, via two possible mechanisms: i) the activation of ab7A induce an avoidance behaviour; ii) the activation of ab7A induce an inhibition of the activation of host-detecting classes of ORNs.

4.4.3.2 The activation of ab7A decreases attractiveness

The next experiment aim was to activate solely ab7A, in addition to an attractive fruit headspace in a behavioural assay. 6-methyl-5-hepten-2-one was chosen because it is the

most abundant compound detected by females in tomato headspaces identified. This compound was also found in other fruits, but it did not induce an activation of ab7A as part of the whole fruit headspaces (Chapter 3). Furthermore, 6-methyl-5-hepten-2-one was previously associated with avoidance by insects (Bruce and Pickett, 2011).

Using strawberry and methyl propionate as attractive fruit and chemical baits, their headspaces were supplemented with 6-methyl-5-hepten-2-one in a 4-choice cage assay. A reduced attraction was visible for fruits implemented with 6-methyl-5-hepten-2-one. Two capillaries of different diameters were used in order to deliver two amounts of the compound in addition to strawberry. A reduction of attractiveness was noticed with the larger capillary, hence when the larger amount of the compound was released. The decrease of attractiveness was significant in all experiments when verified with a Poisson GLM on the number of females found on the supplemented bait compared to the one containing only an attractive headspace (Figure 4-10, Appendix 6).

It can be concluded that an activation of ab7A is associated with a reduction of attractiveness and its ligands can be considered as candidate masking cues. Unfortunately, the control baits (strawberry and raspberry) were not as attractive as expected for host fruits in comparison to previous experiments (Chapter 3) and time constraints prevented from reproducing the experiment.

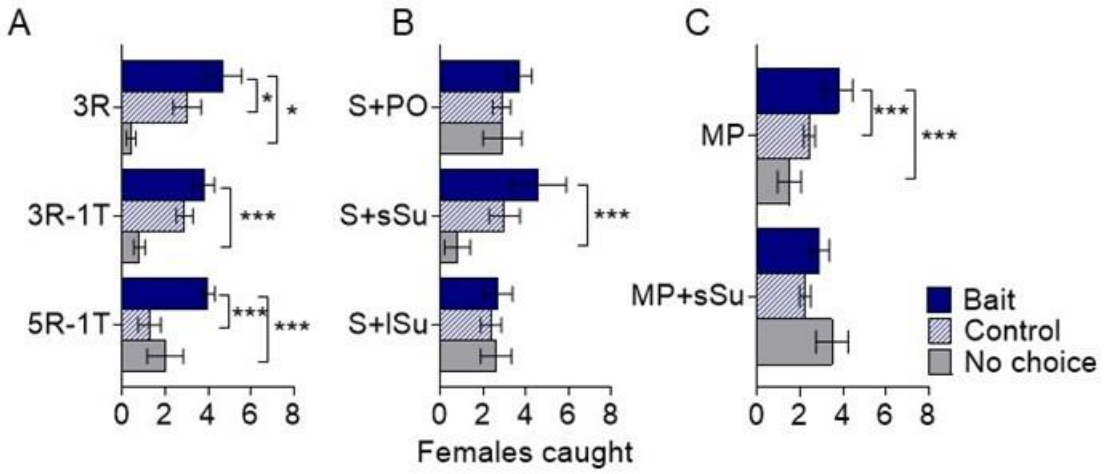


Figure 4-10. Masking host odours by activation of ab7A

Mean number (± SEM) of females collected in a 4-choice cage assay on bait, controls and cage (no choice). The three controls were not different so were pooled and averaged. N=10. GLM with Poisson Distribution: ***, P<0.001, ** P<0.01, *P<0.05. Numbers on the bait were significantly different from control and no Choice, unless indicated (Appendix 6), N=10 replicates with 10-20 females. A) Baits are three raspberry (3R), three raspberry and one tomato (3R-1T) and five raspberry and one tomato (5R-

1T). B) Baits are strawberry with paraffin oil (S+PO), strawberry and sulcatone in a narrower capillary (S+sSu) and with a capillary of larger diameter (S+lSu). C) Baits are Methyl propionate (MP), methyl propionate with sulcatone in a narrower capillary (MP+ sSu). Data for the bait MP are originating from Figure 4-8.

4.5 DISCUSSION

The previous chapter led to the conclusion that host and non-host fruits are detected via fruit-specific patterns of activation or fruitprints on the peripheral olfactory system in *D. suzukii*. Combinations from seven classes of ORNs were involved in host selection. However, some aspects of host selection remained hypothetical and were addressed in this chapter. The headspace volatiles from the six host and non-host fruits (chapter 3) were analysed and tested with electrophysiological and behavioural studies on gravid female *D. suzukii*. The following hypotheses were addressed i) host fruits are selected via the detection of specific and shared volatiles enabling the females to discriminate between multiple hosts; ii) Multiple classes of ORNs are necessary to induce attraction and discrimination of host fruits (Figure 1-4).

The results of this chapter permit to conclude that the peripheral olfactory system encode host odours using multiple classes of ORNs in different combinatorial patterns instead of a single olfactory circuitry (or single class of ORNs) to recognise host fruits.

Two or more classes of ORNs are recruited with at least 40-50 impulses/s increase from the spontaneous activity to induce a behavioural response. It also appears that multiple combinations may induce a similar behavioural outcome. Lastly, the present study demonstrates that fruitprints on the peripheral olfactory system contributes to a model of olfaction which can be used for the identification of semiochemicals for pest management. Fruit volatiles which induce strong responses of host activated ORNs may be candidate attractants, while fruit volatiles which induced strong responses of non-host activated ORNs, ab4A, ab7A and abXA may be candidate masking odours. The findings and their implications are discussed below.

4.5.1 Detection of host fruit specific signals

As a generalist, *D. suzukii* appear to discriminate taxonomically diverse fruits instead of recognising a shared characteristic from all hosts. Based on the analysis of whole ripe fruit volatiles, it appears that attraction to ripe fruits in *D. suzukii* is not mediated by common cues shared by all hosts such as yeast-associated volatiles (Becher 2012). Furthermore, generalism was suggested to be a type of specialism because the insect would have specialised onto several substrates by a physiological adaptation (Loxdale *et al.* 2011). The

fruit-specific pattern of activation of classes of ORNs on the peripheral olfactory system in *D. suzukii* indicate that multiple olfactory circuitry mediate attraction to fruits. Females may recognise diverse hosts because they have an olfactory system that is tuned to a larger selection of chemicals. These results may thus support the above hypothesis.

The present study demonstrated that despite distinct volatile profiles, the three hosts commonly released many chemicals that are ligands for ab1A, ab2A, ab3A and pb1A, the host activated ORNs. The recognition of taxonomically diverse host appears therefore to be via the detection of multitude of fruit-specific chemicals (by multiple combinations of these host-activated ORNs) rather than the detection of a common cue shared by all hosts (via a single olfactory pathway).

4.5.1.1 Characteristics of host fruit volatiles

The chemical composition of the fruit headspace generally matches the ones of earlier reports (Kazeniac and Hall 1970; Hirvi and Honkanen 1983; Larsen *et al.* 1991; Miszczak *et al.* 1995; Bylaite and Meyer 2005; Lasa, Toledo-Hernández, *et al.* 2019). However, this study only focused on a subset of up to 20 chemicals, that are detected by gravid females *D. suzukii*. Particularly, the bouquet of chemicals detected by *D. suzukii* from strawberry matches earlier studies which also looked at which headspace volatiles were detected by this fly (Revadi *et al.* 2015; Abraham *et al.* 2015). A few differences were noted however with small molecules such as the esters methyl acetate, ethyl hexanoate or methyl propionate which have been identified in earlier studies with ripe fruit headspaces and were associated with attractiveness. These were missed in this study but tentatively identified and reported for further studies (Appendix 5). Reasons could be the use of different techniques of extraction of chemicals (Rambla *et al.* 2015) and the use of whole undamaged fruits in the study and not in others.

Host specialisation in *D. suzukii* was hypothesised to be associated with an increased sensitivity to ripe fruit volatiles such as esters following a phylogenetic study by (Ramasamy *et al.* 2016) and functional studies (Revadi *et al.* 2015, Keesey *et al.* 2019). This study also shows that the common detection of esters from strawberry, raspberry and blueberry did not systematically led to attraction. Notably, blueberry was not as attractive as the two other hosts (Chapter 3) despite its large release of esters. Furthermore, the non-attractiveness of orange headspace is not explained by its chemical composition. Indeed, 50% of orange headspace content are esters yet, the orange fruitprint was distinct from the one of host fruits and the headspaces were not attractive (chapter 3).

Overall these results demonstrate that hosts are not recognised by a common cue or characteristic hence, refute the hypotheses stating that hosts are recognised by shared blend of chemicals, and by the activation of common ORNs (Figure 1-4).

4.5.1.2 Recognition of host fruits

Following Sachse and Besher (2016), dedicated ORNs and their innervated glomeruli are tuned to chemicals of similar structures and generally belonging to the same chemical classes. The host activated ORNs, if tuned similarly in *D. suzukii* and in *D. melanogaster*, are largely responsive to many esters and innervated attraction mediating glomeruli. On the contrary, fruit headspaces which major compounds are aldehydes, may activate specific glomeruli and may lead to avoidance in *D. melanogaster*. The present study supports these association as host fruits released mostly esters, while non host released mostly aldehyde and alcohols, which are ligands for the ester-tuned, aldehyde-tuned and alcohol-tuned classes of ORNs respectively. However, these discriminations by chemical classes are not a mechanism of host selection in *D. suzukii*.

Citrus fruits are attractive and favoured host for Drosophilids (Dweck *et al.* 2013) but are not attractive to *D. suzukii*, when undamaged. The latter is sensible as the skin cannot be punctured by females (chapter 3). Nonetheless, the similarity in olfactory recognition of orange with host fruits may be a vestige from the host shift in the *suzukii* lineage as citrus fruits are favoured fruits for *Drosophila* (Dweck *et al.* 2013).

Blueberry is one of the most infested crops together with raspberry and strawberry (EPPO 2019). Its weak attractiveness compared to the two latter was measured and discussed in chapter 3. The present study demonstrates that the low attractiveness of blueberry observed is the result of low amounts of released ligands for the host-activated classes of ORNs, not the result of the activation of other classes as it is the case for non-hosts. Additional research is needed to identify the mechanism of recognition of blueberry. The release of attractive chemical is too low, perhaps because of postharvest condition of the fruit (Boschetti *et al.* 1999). Furthermore, attractiveness in the field may be because of semiochemicals released by other plant materials and associated organism (Figure 1-4). Volatiles that were tentatively identified from other plant parts (e.g. foliage) in this study may contribute to an increased attraction to blueberry bushes in field conditions. The whole plant may be a source of signals to locate ripening fruits. Notably the foliage may release chemicals that are detected and attractive to *D. suzukii* (Keesey *et al*. 2015). Furthermore, different plant parts such as flowers release distinct scents from ripening fruits (Robertson *et al.* 1995). *D. suzukii* may detect flower specific volatiles indicating that no ripening fruits are available or perhaps as a feeding attractant. Headspace from ripe fruits on their plant,

the foliage and flowers and whole plants were collected and tentatively identified (Appendix 5). Unfortunately, the study could not be completed. Few GC-FID peaks differed for the foliage profiles, but the chemicals and their bioactivity were not determined.

4.5.2 Multiple olfactory circuitries mediate attraction to host fruits

A dedicated olfactory system for host selection in *D. suzukii* consists in seven ORNs classes (chapter 3). Each host fruit activated specific and overlapping classes suggested that they can be discriminated. Which and how many of the fruit-activated classes of ORNs are recruited to induce a behavioural response? The results of this chapter demonstrate that not one single ORN class is involved in mediating attraction to hosts but multiple combinations of several ORNs mediate attraction, suggesting that multiple olfactory circuitry can mediate a same behavioural outcome, supporting results from Chapter 3 and defending the hypotheses addressed in this thesis (Introduction, 1.4). The results of this chapter led to the following hypothesised mechanism. They are discussed but additional work will be needed for validation.

It can be hypothesised that each olfactory circuitry that has encoded different stimuli (e.g. different chemicals) may induce different types of attraction, from weak to strong: i) a common combination to all fruits may be activated (ab1A and pb1A were commonly activated by the three hosts); ii) attraction may be increased with recruiting addition host-activated ORNs (ab2A and/or ab3A); iii) reversely, the recruitment of non-host activated ORNs may decrease attraction (ab7A, ab4A and/or abXA).

4.5.2.1 One class of ORN is not sufficient to mediate attraction

In this study the simultaneous activation of the classes ab1A and pb1A induced attraction but when tested alone or in different combinations, ab1A was not sufficient and pb1A was not necessary. These results contradict earlier hypothesis formulated by which the olfactory system is composed of single ORNs dedicated to behavioural outcomes in *Drosophila* (Hansson and Christensen 1999; Hansson and Stensmyr 2011; Sachse and Beshel 2016) and as discussed in chapter 3.

The ab1A which was commonly active by all hosts, mediate attraction, but its sole activation was not sufficient. The association of ab1A with attractiveness is supported by high responses to host fruit headspaces in *D. suzukii* (chapter 3) and to most fruits in *D. melanogaster.* The genes coding for Or42b (associated with ab1A) were found conserved in *D. suzukii* compared to *D. melanogaster* (Hickner *et al*. 2016; Ramassamy *et al.* 2016). In this study, the function of ab1A was not different between species (Chapter 7) and the number of sensilla on the antenna was similar, contrary to the ab2 and ab3 types. These

observations were also made by Keesey *et al*. (2019). It suggested that ab1A is associated with attraction to fruits in Drosophilids.

The addition of pb1A to ab1A mediated attraction yet, attraction was also induced by isoamyl acetate, without activation of pb1A indicating that it may not be necessary. The behavioural response to a sole activation of pb1A was not tested therefore, its sufficiency is not known. A small response of approximately 50 impulses/s appeared associated with attraction to methyl propionate and raspberry (chapter 3). Furthermore, another attractive substrate, the grape cultivar Schiava released volatiles that are ligands for pb1A, unlike unattractive grape cultivars tested in the same study (chapter 5). The class pb1A is also one of the most functionally divergent ORN classes between *D. suzukii* and *D. melanogaster* suggesting an important role in ripe fruit detection and host selection (chapter 6). It is further supported by an expansion and two novel isoforms of the *Or42a* gene coding for the Or42a associated with pb1A in the Suzukii lineage (Ramassamy *et al*. 2016; Hickner *et al*. 2016).

At least two classes of ORNs must be activated to induce a behavioural response following the results of this study: for instance, the ab1A was attractive only when activated simultaneously with pb1A. This attraction was similar to one from strawberry headspaces (approximately 50% more females were found on the odour bait than on control).

4.5.2.2 Increased attraction with combinations of ORNs

Ethyl acetate was found attractive in wind tunnel but not in the 4-choice cage assay where only one class was activated. Unfortunately, it is unclear which classes were activated by the chemical bait in the wind tunnel. The reported attractiveness of ethyl acetate may be achieved at higher doses, when more ORN classes are activated. Indeed, the classes pb1A, ab2A and ab3A are also responsive to ethyl acetate in *D. suzukii* in this study and, the recruitment of the DM1, DM2, DM3, and DM4 glomeruli in *D. melanogaster* led to increased attraction in earlier studies (Thoma *et al*. 2014, Mohamed *et al.* 2019, Munch and Galizia 2016). A recruitment of additional ORNs, OR and glomeruli may occur as the dose of chemical detected increases and may lead to either increased attraction (as above), or decreased attraction (Malnic *et al.* 1999; Stensmyr *et al.* 2003; Wang *et al.* 2003; Semmelhack and Wang 2009).

4.5.2.3 The ab3 is involved in attraction but is not necessary

This study supports a role of ab3A in host selection, in combination with other ORN classes. First, ab3A was commonly activated by headspaces of three attractive host fruits (Chapter 3) and most ripe fruit headspace compounds were identified as high affinity ligands for ab3A. A loss of function, originally identified for *Or22a*, associated with ab3A (Table 1.1)

was directly linked to a reduced detection of fermenting odours in *D. suzukii* compared to other Drosophilids (Ramassamy *et al.* 2016). It functionally diverged from ab3A in *D. melanogaster* according to Chapter 6 and a study by Keesey *et al.* (2019). Furthermore, ab3A was associated with host specialisation in several studies in *D. suzukii* (Keesey *et al.* 2015) and other Drosophilids, including *D. orena* (Comeault *et al.* 2017), *D. sechellia* (Dekker *et al.* 2006) and *D. erecta* (Stensmyr *et al.* 2003). The ab3A was also associated with *D. melanogaster* attraction to the ancestral host fruit (marula) via detection of ethyl isopentanoate (Mansourian *et al.* 2018). The latter compound was identified as a major component of blueberry fruit headspace with a release rate of about 400 ng/h for 100g of fruit. As blueberry did not induce high attraction of *D. suzukii* in chapter 3 despite its headspace activating ab3A and pb1A. Blueberry also released many other esters that are ab3A ligands (Figure 4.2 & 4.3). It can be suggested that the activation of ab3A may be too low or the activation of additional ORNs is too low, notably from ab1A and ab2A to induce a high attraction from these blueberry fruits used, as discussed above and chapter 3. Time constraints prevented from testing the behavioural response resulting from activating only ab3A.

Additional findings suggested that even though ab3A may be associated with host specialisation and attraction may not be as necessary as believed. Notably, the impaired detection of β-cyclocitral by ab3A did not change the fruitprints (Chapter 7). This compound was associated with attraction via activation of ab3A in *D. suzukii* by Keesey and colleagues (2015) in field assays using yeast-based trap. It was also found to enhance attraction of fermenting fruit baits by Pinero *et al.,* 2019. In both studies, it is unlikely that ab3A was the only activated ORN in their tests given that several chemicals are released from yeast baits that are attractive to *D. suzukii*. Furthermore, the present study revealed that β-cyclocitral was only found in orange headspaces, hence not associated with the attraction to host fruits observed. Lastly, attraction was induced in a behavioural assay without activating ab3A. It shows that both ab3A and β-cyclocitral may be involved in host selection but are not necessary.

4.5.2.4 The ab2A is involved in attraction but is not necessary

As discussed in chapter 3, the role of ab2A is unclear, and the present study showed that its activation was not required to induce attraction. The ab2A was highly activated by the most attractive fruit headspaces and is in the most abundant functional sensillum type on the antenna of *D. suzukii* (chapter 3). and its associated ORs were positively selected in the Suzukii lineage (Hickner *et al.* 2016; Ramasamy *et al.* 2016; Keesey *et al.* 2019).

4.5.3 Combinations of ORNs to disrupt attractiveness

Non-host fruit headspaces of grape, orange and tomato were characterised by their fruit specific ORN activation of ab4A, ab7A and abXA, the latter being also activated by the attractive strawberry headspaces. From these headspaces, a minority of ligands induced responses of host activated ORNs and were found in the smallest amounts. It suggested that the lack of attractiveness of these fruits compared to host fruits may not be caused by a too low amount of host activated ORNs (as for blueberry, see above). The non-attractiveness of grape, orange and tomato headspace is likely due to the additional ORNs activated.

Is it possible to disrupt attraction to host fruits by activating these ORNs? This may be a mechanism by which non-host fruits are avoided. It could also be used to disrupt attractive signals from host fruits in infested orchards.

Having mixed both attractive (raspberry) and unattractive signals (tomato) may have confused the fly, hence attractiveness was reduced. This disruption of attractive signals was likely mediated via the increased activation of the ab7A and ab4A neurons, simultaneously with raspberry-activated ab1A, ab2A and ab3 (A and B). Here the two sources formed only one, so they were simultaneously detected: the attractive host was no longer detected because the combination of ORNs changed and did not mediate attraction (Chapter 3). This result is supported by earlier studies: the dynamic and timing of detection of attractive and unattractive sources was determinant for *D. melanogaster* which could not tell apart the two sources if they were simultaneously presented (Leal 2013; Larter *et al.* 2016).

4.5.3.1 The role of ab7A

6-methyl-5-hepten-2-one is a ligand of high affinity for ab7A and is the most abundant bioactive compound from tomato headspaces (Figure 4-1) a non-host, non-attractive fruit (chapter 3). This compound has been associated with plant stress caused by insect feeding damage (Quiroz *et al.* 1997) and may thus be produced by these fruits because they have been harvested thus technically damaged. It was suggested by Bruce and Pickett (2011) following a review of host-insect chemical interactions that plants releasing high amounts of chemicals associated with damages could be considered of lower quality by phytophagous insects, hence be avoided. This compound reduced the attractiveness of fruit and chemicals when supplemented in several behavioural assays supporting the masking effect of activating ab7A, but the decrease was not very strong. This may be due to a lower attraction from the fruits in control trials, which cultivars and condition of experiment may have changed from earlier experiments. However, this compound is also a ligand of ab2B but not for ab4A (as tomato headspaces activated). It is therefore unclear whether the

masking effect obtained was weakened by the activation of ab2B, or because of the absence of activation of ab4A. The ab7A might not be sufficient to mask attractive odours or induce avoidance. Additional studies are required to specifically look at the activation of ab7A, notably using genetic ablation to activate or inhibit solely ab7A. Such tools are currently investigated in *D. suzukii* (Mansourian *et al.* 2019).

Tomato headspace also activated ab4A, which may have worked together with ab7A. In addition, an experiment testing the two enantiomers of Linalool (discussed above) indicated that R linalool may have an inhibitory effect on an attractive mixture containing (S)-linalool and methyl propionate (also attractive alone). The comparison of (S)- and (R)- linalool detection showed that the two isomers activated distinct combinations of ORNs, involving an activation of ab7A and of abXA by the non-attractive mixtures. Ab7A was therefore never activated on its own. A specialization of ab7A for attraction or repellence as for other ORNs (such as host-activated ones) was not determined. Under the assumption that olfactory units are arranged similarly in *D. suzukii* and in *D. melanogaster* (Introduction, 1.3) the activation of glomeruli VM5v innervated by ab7A may therefore be inhibiting the glomeruli innervated by host activated ORNs. Crosstalks between glomeruli were also demonstrated by Semmelback and Wang (2009): DL1 innervated by ab4A (also activated by tomato headspaces mediated a reduction of attraction by a crosstalk with attractant-responsive glomeruli and have shown to disrupt attractiveness in *D. melanogaster* (Hallem and Carlson 2006, Munch and Galizia 2016, Knaden *et al.* 2012, Mohamed *et al.* 2019).

4.5.3.2 The ab4A may be associated with ripeness

The activity of ab4A decreased when the decaying process of grape was advanced by 48h (Chapter 3). This indicated that the sole ligand for ab4A identified from grape headspaces, (E)-2 hexenal levels decrease with ripeness and are reported with postharvest aging in grapes (Kalua and Boss 2009). Furthermore, similar aldehydes (notably hexenal and (Z)-3-hexenal) and their corresponding alcohols were associated with defense mechanisms of plants against pathogens (Bate and Rothstein 1998), suggesting that they may be an indicator of unhealthy substrates for *D. suzukii*. They were also mostly released by non-attractive fruits in this study but not by attractive undamaged fruits, results supported by earlier studies (see above). This is supported by findings of Myung *et al.* (2006) in which (E)-2-hexenal is only produced by strawberry upon wounding. It can thus be suggested that fruits which headspaces do not activate ab4A are more attractive to *D. suzukii* hence, ab4A is associated with non-attraction. In addition, (E)-2-hexenal elicited repellency in *D. melanogaster* via activation of ab4A and its innervated repellent-responsive glomeruli DL1 (Hansson et al., 2010, Mohamed et al 2019).

However, Or7A (associated with ab4A) which is responsive to (E)-2-hexenal was also associated with aggregation behaviour. It showed to mediate attractiveness to oviposition substrates via detection of the pheromone 9-tricosene in female *D. melanogaster* (Lin *et al.* 2015). The ab4A may therefore mediate different behavioural outcomes depending on which ORNs it is simultaneously activated with, as for ab2B.

It can be concluded that ab4A may have a role in host discrimination from non-host when simultaneously activated with other ORNs. The combination may determine whether attraction (with ab1A or other host activated ORNs) or avoidance (with ab7A) is mediated. This hypothesis may need further development by behaviourally testing the activation of single and multiple ORN classes.

4.5.3.3 The dual role of ab2B

Did ab2B reduced the masking effect expected from ab7A as hypothesised before? The role of ab2B remained unclear from these results. As for ab4A, ab2B may be associated with different behavioural outcomes depending on the combination it is associated with. In *D. melanogaster* the ab2B neurons innervate both DM3 and DM5 glomeruli which are associated with attraction and aversion respectively (Laissue and Vosshall, 2008; Semmelhack and Wang, 2009). This dual role is supported by results of this study: it can be hypothesised that ab2B is involved in two behavioural pathways depending on which combination it forms with other ORNs, notably ab3A and ab7A.

First, the non-attractive tomato and grape headspaces did not induce a simultaneous activation of ab7A with ab2B (chapter 3) but the major compounds are ligands for both ab2B and ab7A neurons. Other chemicals that are associated with non-attraction and possibly aversion, are (R)-linalool and nepetalactol (Chapter 6) which induced a high activation of ab2B (> 100 impulses/s) in *D. suzukii*.

Secondly, the ab2B may also be part of an olfactory circuitry inducing attraction when simultaneously activated with ab3A. Indeed, ab2B was activated by the attractive isoamyl acetate in a behavioural assay. Furthermore, many ligands of high affinity for ab2B were identified from attractive hosts strawberry and raspberry. These chemicals are associated with ripening fruits and likely to be attractive cues for *D. suzukii* (Keesey *et al*. 2015; Ramassamy *et al*. 2016; Revadi *et al.* 2015; Abraham *et al*. 2015). Many of these chemicals are also ligands of high affinity for ab3A. This role in attraction is further supported by ab2B being housed in the most abundant functional sensillum type ab2 as demonstrated in chapter 3 and reported by Keesey *et al.* (2019). In addition, an activation of ab2B by strawberry and raspberry headspace was hypothesised but due to the high activity of ab2A, it is unclear whether the neurons were activated and masked, or on the contrary inhibited

(chapter 3). It is possible that ab2A have inhibited ab2B activation. This modulation of signals was found between co-localised ORNs in *D. melanogaster* (Dobritsa *et al.* 2003; Goldman *et al.* 2005; Su *et al.* 2012; van der Goes van Naters 2013). In addition modulation of responses is also possible between postsynaptic partners via PNs (Gorur-Shandilya *et al.* 2017) and could be affect the responses of the two ORNs or their innervating glomeruli.

4.5.4 A threshold of activation to mediate a behavioural response

How much activation is needed to trigger a behavioural response? To date little is known about the amount of activation required for classes of ORNs to mediate a behavioural response. Kaissling (2009) has specifically looked at the activation-induced behaviour in *Bombyx mori*, and found that about three times the standard deviation of the spontaneous activity of the neurons was behaviourally active. In *D. melanogaster* behaviourally relevant activation was considered with an increase of twice the spontaneous activity (Dweck *et al.* 2018).

The present study suggests that an increased in the activation of 40-50 impulses/s compared to the spontaneous activity suffice to activate the ORN in a combination involving multiple classes of ORNs but was not measured. In several behavioural assays, activations of approximated 40-50 impulses/s (including the dilution in the airstream during the SSR experiment) were associated with a behavioural response: The pb1A was activated with approximately 40 impulses/s and appeared as a sufficient addition to the activation of ab1A to induce attraction; the activation of abXA with approximately 50 impulses/s appeared associated with a decrease in attraction. Furthermore, the statistical distinction between ORN activations in chapter 3 and chapter 6 demonstrated that ORNs with the highest activation, forming a characteristic fruitprint were all above 40 impulses/s. Notably, raspberry headspaces are one of the most attractive fruit for *D. suzukii* and activated pb1A with approximately 45 impulses/s only. (chapter 3). Considering that the headspaces were diluted about 10-fold in the airstream when recording responses, it is possible that the actual activation in the behavioural assay was higher. Hence, it is not possible to predict real threshold value of activation from these results. How the behavioural response varies with the intensity of activation of ORNs remain to be deciphered. The additional modulation of olfactory signals in the antennal lobe and other centralised olfactory units would also need consideration.

4.5.5 Prospects for pest control

4.5.5.1 A model for the development of management tools

The impact of this research is to contribute to the discovery and use of novel tools for integrated pest management (IPM) strategies against *D. suzukii*. Using single cell recording (SSR) the present study enabled an increased sensitivity and precision in the identification of behaviourally active compounds compared to the use of whole antennal responses (EAG). Using the fruitprints identified in chapter 3 it is possible to identify which classes of ORNs mediate attraction. Fruitprints on the peripheral olfactory system contribute to a model of olfaction which can be used for the identification of semiochemicals for pest management.

Earlier studies on other insects demonstrated that it was possible to create a very attractive chemical bait using compounds identified from host odours. The identification of whole antennal (EAG) response enabled the selection of candidate attractant and use of attractive host odours to develop species specific bait have been successful against the tsetse fly (Diptera: *Glossina spp*), vector for Trypanosoma sp. Attractive odours, notably (±)-1-octen-3-ol, from cattle were used in traps and significantly attracted flies in field trials (Hall et al., 1984; Burssell et al., 1988; Torr et al., 1996, 1997). Other example is the use of ripe fruit host volatiles against several fruit fly species such as the oriental fruit flies (Alyokhin *et al.* 2000), African fruit fly (Biasazin *et al.* 2014) or the apple maggot *Rhagoletis pomonella* (Cha *et al.* 2017). For instance, attractive compounds were identified from host because they induced high antennal responses and induced strong behavioural responses. Most recently, populations of *Rhagoletis* flies infesting snowberry fruits were found to be significantly attracted to a 9 components-blend of host fruit volatiles, identified from the snowberry. Two key host fruit volatiles: (E)-4,8-Dimethyl-1,3,7-nonatriene (DMNT) and (±)-1-octen-3-ol were necessary in this blend to induce significant attraction in flight assays (Cha *et al.* 2017). These two fruit volatiles were also detected by *D. suzukii*. The classes of ORNs they activate are however not associated with attraction.

4.5.5.2 Prospects for a Push-pull design with ripe fruit odours

A mechanism by which masking or repellent odours are processed was identified enabling to identify repellent or masking semiochemicals. Host plants may be masked or rendered unattractive by using masking or repellent semiochemicals (identified from nonhosts) in addition to alternative attractive lures around the crops: lures and traps made from ripening fruit volatiles. This technique known as Push-Pull, is effective in many crop systems (Cook *et al.* 2007) and showed promising results against *D. suzukii*, in highly infested raspberry crops (Wallingford *et al.* 2018).

In addition, Cha *et al*. (2019) measured a decrease of attractiveness from raspberry odours infested with a phytopathogen, *Botrytis cinerea* Pers. The identification of the ORN classes that are differentially activated by infected and non-infected fruits would enable to decipher a mechanism of odour masking which would be very valuable for the development of pest management tools. The masking of raspberry and strawberry odours was attempted in this study using the activation of ab7A. The results are promising, and additional work is required to fully characterise their roles.

4.5.5.3 Support for slow release of semiochemicals

Candidate semiochemicals have been found in this study. Their pattern of activation on the olfactory system highlighted which are candidate attractant or possible repellent depending on which ORN they activate. The identification of the ORNs whose activation may induce an inhibition of attraction may enable the identification of candidate masking odours to be used as a means of disruption among crops. Behavioural tests will enable the design of attractive or repellent mixtures for *D. suzukii*. It requires a suitable release of chemicals for them to activate the desired combination of ORNs in the bioassay and in the field. Capillaries appear to be a dispenser that is difficult to use and time consuming, with more than one chemical. The following alternative are interesting for further development in traps for *D. suzukii*.

Rubber septa have been used successfully as pheromone dispensers for mating disruption of Lepidopteran agricultural pest (Knight 2002). However, pheromones are not as highly volatiles as esters and alcohol from ripe fruits which may thus not be retained as easily by the septum. Assays with isoamyl acetate in this study and by Revadi *et al.* (2015) were successful for laboratory trials suggesting that alcohols or esters may also be used. Sprays have also been used with *D. melanogaster* and proven an effective constant release system to test long range flight behaviour (Becher *et al.* 2010).

Polyethylene sachets, as used for tsetse flies (Torr *et al*. 1997) and is currently being tested for the control of aphids (Pers. Communication from J. Pickett, Cardiff University, UK) appear suitable for slow release of ripe fruit volatiles in field trials. A screening of different mesh sizes and release surface area is however needed to apply a release rate which would induce attraction or a masking effect of host odours.

4.5.5.4 Isoamyl acetate

Isoamyl acetate attracted females in both the present study and one conducted by Revadi and colleagues (2015). A dose of 10 µg of isoamyl acetate loaded on a rubber septum gave a similar release rate than fresh fruits estimated at about 200 ng/ h (Revadi *et al.* 2015). In the present study, isoamyl acetate was released at an approximate rate of 100 ng/h (per

100g fruit) estimated from strawberry headspaces (Figure 4-1). Its attractiveness in the wind tunnel also supports the findings from Revadi and colleagues. Based on the assumption that isoamyl acetate activated the same classes of ORNs in the behavioural assay as when a similar dose was tested in SSR (30 µl of a 10^{-2} g/ ml solution on filter paper) the behavioural response appears associated with an activation of many classes of ORNs (Figure 4-10). Some of the genes coding for the associated ORs underwent evolutionary changes, the most notable being *Or67a*, co-expressed with ab10A which was quadruplicated (Revadi *et al*. 2015). These ORNs were however not activated by fruit headspaces suggesting other roles of importance for *D. suzukii*.

Which ORNs are mediating attraction of *D. suzukii* to isoamyl acetate in a behavioural assay? The present study demonstrates that ab2B and ab3A were the most sensitive classes of ORNs to isoamyl acetate and may be the first most activated by isoamyl acetate from fruit headspaces. However, more classes, including ab1A and ab7A were activated by isoamyl acetate at a higher dose and may have been activated in the behavioural assay. An interesting consideration is the reduced attractiveness with increased dose reported by Revadi *et al.* (2015). It suggests that the additional ab7A in addition to ab2B may have inhibited the other ORNs (as discussed above).

4.5.5.5 Disruption of attraction to isoamyl acetate

The attraction to isoamyl acetate in a wind tunnel may have been decreased because of i) a reduction of ORN activity due to its lower amount; ii) diacetone alcohol forms an unattractive blend with isoamyl acetate and 2-methyl butyl acetate.

Isoamyl acetate and 2-methyl butyl acetate activated the same ORNs with similar intensity (at the dose tested of 0.01%). A change in attractiveness caused by the blend of the two is possible if the two compounds are agonists. Mixtures of isoamyl acetate with other compounds have been found to be less attractive than isoamyl acetate alone suggesting interactions that are significantly affecting the detection of this compound (Cha *et al.* 2017; Cloonan *et al.* 2019; Piñero *et al.* 2019).

Another hypothesis is that diacetone alcohol may have induced a masking or repellent effect when added to the blend. It appeared as an active semiochemical in other studies: it was found as an oviposition attractant for the polyphagous pest *Delia platura* on bean plants (Gouinguené and Städler 2006). This compound is also released by damaged bean plants and attract the parasitoid *Dyglyphus isaea* Walker, a predator used for biological pest control against the leaf miner *Liriomyza trifolii* Burgess (Finidori-Logli *et al.* 1996). The composition of the blend was unexpected and requires further characterisation before to consider diacetone alcohol as a candidate semiochemical for pest management. Indeed, it

is used in certain insecticide formulations and was found to be highly toxic to small birds (Kitulagodage *et al.* 2008).

4.5.6 Detection of mixtures and single components

Behavioural experiments led to unexpected findings that are discussed below. Combination of compounds induce different activations than the compounds alone, as previously observed in other species (Bruce and Pickett 2011). The present study demonstrated that several classes of ORNs needed to be activated to induce a behaviour. Furthermore, a mixture of two enantiomers induced distinct behaviours and activation than if tested alone. Responses to mixtures are also governed by crosstalks and interactions between olfactory units (Silbering and Galizia 2007; Mohamed *et al.* 2019) which renders difficult the study of naturally occurring odours such as fruit headspaces as illustrated above. Conflicting results of attraction and avoidance being mediated by the same channels highlight the complexity of signals detected from fruits in the environment.

4.5.6.1 Enantiospecificity

The present study show that the two enantiomers of linalool activated specific combinations of different and overlapping classes of ORNs in *D. suzukii*. The pattern of activation of ORNs by the mixture of the two enantiomer (*RS*) also does not reflect the pattern of each enantiomer presented alone. In addition, a combination of several host and non-host activated ORNs are simultaneously activated by Linalool. The addition of (R)-linalool decreased the attractiveness of a bait in a behavioural assay was associated with an increased activation of abXA. The role of the abXA neurons is unclear as it was strongly activated by both an attractive host and an unattractive fruit (Chapter 3). (S)-linalool was the only enantiomer which was identified from fruit headspaces (host and non-host) and in the largest amount in attractive fruits (e.g. strawberry). Linalool have been found to act both as a host plant attractant or as a repellent. These behaviours were showed associated with a differential detection of the two enantiomers. in the weevil *Anthonomus rubi,* different specialised olfactory receptors are activated: one receptor detects (R)-Linalool and a different receptor detects (S)-Linalool (Bichão *et al.* 2005). They can also be detected by the same receptor with different sensitivities, like in the moth *Mamestra brassicae* (Ulland *et al.* 2006).

4.5.6.2 Detection of odour plumes

As discussed in chapter 3 it is still unclear how females perceive and decrypt information from odour plumes hence, it is difficult to design an assay in which the odours would be release in natural like condition. The wind tunnel appeared as most realistic representation

of a natural environment, with a directional wind, which females may use for orientation, as showed by Suver *et al.* (2019). Insects orient towards the source of the plume using the dynamic and detection of the odours within the plume (Murlis and Jones 1981).This directional behaviour was also described in *D. melanogaster* towards odours of ripe banana (Budick and Dickinson 2006). The response to fruit odours in a wind tunnel appeared more direct and stronger than in a 4-choice cage assay suggesting gravid females *D. suzukii* may use the directional wind as a cue (Chapter 3). Furthermore, the olfactory detection of odour plumes combines the activation of ORNs from the chemical composition of the plume, the physical structure and the temporal distribution of odour molecules (Elkinton and Cardé 1984; Vickers *et al.* 2001; Lei *et al.* 2009).

4.6 CONCLUSION AND FUTURE DIRECTION

The results of this chapter demonstrate that the peripheral olfactory system in *D. suzukii* encode fruit odours as follow: each fruit headspace is detected as a fruit-specific bouquet of volatiles with little overlapping shared by host fruits. Each fruit type appears to be attractive because of combinatorial patterns of activation involving two or more shared and distinct ORNs. Distinct patterns mediate attraction demonstrating that multiple olfactory circuitries can mediate attraction hereby enabling discrimination of taxonomically diverse hosts.

The present study describes the pattern of activation of ORNs on the peripheral olfactory system by 55 fruit volatiles which may inform on their potential roles as attractant or repellent. These may be further tested with behavioural assays and perhaps developed into attractive lures or repellent/masking cues to disrupt host selection in commercial crops.

As discussed, additional research is needed to deepen the understanding of how the encoding of fruit odours drives host selection in generalist species that are thriving in various habitats.

The following questions are addressed in the next chapters of this thesis: Is a similar model of olfaction enabling females *D. suzukii* to distinguish suitable oviposition sites from several cultivars of a same plant species (Chapter 5)? How did the encoding of ripe fruit odours evolve in the *Suzukii* lineage, notably from an overripe fruit/ yeast specialist that is the closely related *D. melanogaster* (Chapter 6)?

CHAPTER 5

5 CASE STUDY ON WINE GRAPE CULTIVARS: HOST STATUS IS DETERMINED BY HEADSPACE COMPOSITION

Adults *Drosophila suzukii* on a grape berry (*Vitis vinifera*)

5 VALIDATION OF THE HOST SELECTION MODEL WITH A CASE STUDY ON WINE GRAPE CULTIVARS

5.1 INTRODUCTION

5.1.1 Aim of chapter

D. suzukii can discriminate between plant species from different families and within families (i.e. Rosaceae) because their odorant bouquet elicits species-specific patterns of activation of ORNs on the peripheral olfactory system of the insect (fruitprint). Cultivars of a same plant species (i.e. strawberry, blueberry) were found to induce similar fruitprints in *D. suzukii*. Yet, cultivars of grape and blueberry fruits differ in their susceptibility for egg laying. How do generalist species discriminate among cultivars of a same plant species? The following hypothesis is addressed: grape cultivars release distinct chemical blends that are detected by subsets of specific and overlapping ORNs, allowing the gravid females to discriminate cultivars with suitable and non-suitable fruits for oviposition.

To assess whether flies can discriminate suitable egg laying sites among cultivars of a same plant species, a fruit type which susceptibility differ per cultivar was ideal. Grapes were a suitable model as fruits of some cultivars were found more susceptible to egg laying than others (see below). Different grape cultivars have also been cultivated for centuries for their unique aromas and flavours. The different aromas come from a variation in the headspace composition of the fruit and may be recognised by gravid female *D. suzukii* searching for egg-laying substrates.

The comparative study using five wine grape cultivars was possible thanks to a kind collaboration with Enzo Mescalchin and Claudio Ioriatti, (FEM, Italy). The comparison showed that Schiava was the only cultivar suitable for oviposition and which headspaces were the most attractive. The attractive volatile compounds which may have driven the preference of *D. suzukii* females for Schiava headspaces were tentatively identified. They were tested for electrophysiological response on the seven classes of ORNs that are activated by ripe fruit headspace volatiles. The identification of repellent or attractive cues that are naturally used by the fly to discriminate among grape cultivars may become candidate volatiles to use for disruption or mass trapping.

5.1.2 Background

5.1.2.1 The peripheral olfactory system of D. suzukii

The olfactory system of *D. suzukii* can be briefly summarised as follows. It consists of olfactory receptor neurons (ORNs) that are housed in sensilla, which are finger-like or hair-

like cuticular structures on the surface of the antennae and maxillary palps. ORNs harbour the olfactory receptors (ORs) which are tuned to few or many different food and host volatiles, in their dendrites. The ORNs mediating host/food odour detection are located in basiconic sensilla and each type of basiconic sensillum houses two to four ORNs (Introduction, 1.3). Approximately, 13 functional types of basiconic sensilla have been identified, labelled ab1 through ab10 on the third antennal segment and pb1, pb2 and pb3, on the maxillary palp. The ORNs can be recognise by the amplitude of their action potentials in extracellular recordings and are labelled according to this amplitude and, together with the other ORNs with which they share a sensillum, define the functional sensillum type (Method 2.8.2). For instance, the basiconic sensillum type ab1 house four ORNs: ab1A, ab1B, ab1C and ab1D. These ORNs relay olfactory information towards the glomeruli of the antennal lobe (Introduction 1.3).

Extracellular recordings (SSR) of the activity of these ORNs allows their response to chemical stimuli to be measured. This technique permitted to characterise the classes of ORNs in *D. suzukii* (chapter 3). Furthermore, the recording of the activity of these ORNs in response to ripe fruit odours permitted to identify that seven of these classes of ORNs encode ripe fruit odours in *D. suzukii* (Chapter 3). These activations were characterised as strong and relevant with more than 50 impulses/s during a stimulus (Chapter 3).

5.1.2.2 Encoding of ripe fruit odours

Ripe fruit odours are encoded by combinations of seven classes of ORNs allowing females *D. suzukii* to discriminate host and non-host fruits for oviposition (chapter 3). Fruit headspace release blends of species-specific volatiles that are detected by *D. suzukii*. Attractive fruits were associated with the activation of two or more of the four classes of ORNs pb1A ab1A ab2A and ab3A (Chapter 3). ab1A and pb1A were shown to be an attractive combination as their simultaneous activation with fruit volatiles induced attraction of gravid females *D. suzukii* (Chapter 4). Unattractive fruits were associated with the activation of ab7A, ab4A and abXA (Chapter 3). Notably, the fruitprint of the grape cultivar sugraone was formed mainly by ab4A, ab7A and a small activation of ab1A. The fruits were not attractive and were not oviposited.

The grape fruitprint is a non-attractive pattern of activation and grapes are not perceived as host fruits by gravid females *D. suzukii*. Grape cultivars that are susceptible are different from other host fruits because fewer eggs are laid in the fruits and few of these successfully develop into adults (Lee *et al*. 2011). Furthermore, grape susceptibility to the fly is closely associated to cultivar specificities (see below).

The class ab1A is associated with attraction to fruit headspaces, yet its sole activation did not induce a behavioural attraction (Chapter 4). The intensity of activation of classes of ORNs vary with cultivar and fruit ripeness (Chapter 3), therefore as discussed in chapter 3 and 4, it can be suggested that an increase in the intensity of the ab1A, coupled with a decrease in the activation of ab7A and/or ab4A may lead to increased attraction in some cultivars. Alternatively, the ab4A may be part of two olfactory circuitries, and its simultaneous activation with ab1A may be more attractive.

5.1.2.3 Field observation of the susceptibility of different cultivars

Grape cultivars have been reported with major damages from *D. suzukii* in wine producing regions (Walsh *et al.* 2011; Rouzes *et al.* 2012; Van Timmeren and Isaacs 2013; Ioriatti *et al.* 2015). Despite not being the most suitable fruit to support larval development, vineyards suffer major damages as the sole puncture of the skin for oviposition attempts induce significant damages (Lee *et al.* 2011; Entling *et al.* 2019). Field studies looking at infestation in vineyard permitted to link the susceptibility of grape cultivars to the physical state and sugar content of the berries. Fruits with thick skin such as the cultivars Yellow Muscat, Traminer or Teroldego, are significantly less damaged than soft skinned varieties such as Schiava, Pinot Noir or Chardonnay (Bellamy *et al.* 2013; Ioriatti *et al.* 2015). In Trentino and Sud-Tyrol provinces (Italy) Schiava vineyards were the most damaged compared to neighbour cultivars (Pers. Comm. from E. Mescalchin, FEM, and F. Sinn, Beratugring, Italy).

Deciphering whether flies can recognise cultivar suitability may provide novel tools to disrupt host attractiveness. Masking or repellent cues from the non-host cultivars may be used to mask the attractiveness of oviposition substrate. This could be used to tackle the infestation in vineyards and potentially be applied to other crops.

5.2 ATTRACTION TO FIVE GRAPE CULTIVARS FOR OVIPOSITION

The first step of this study was to assess the preference among headspaces of five cultivars of wine grapes with different susceptibility to *D. suzukii*: Schiava, Traminer, Merlot, Pinot noir and Lagrein. Using an oviposition assay and a multiple choice experiment the susceptibility and attractiveness of fruits was assessed. The fruit skin was undamaged (i.e. divided by cuts on the stems) and fruits were not visible in the multiple choices assay.

Schiava was the most suitable cultivar for egg laying with 2-5 eggs laid per day. It was the only cultivar with more than one egg laid. The headspaces attracted about 40% of the responding females in the multiple choices assay and was the preferred cultivar (Figure 5-1). It was concluded that Schiava grapes can be categorised as host fruits.

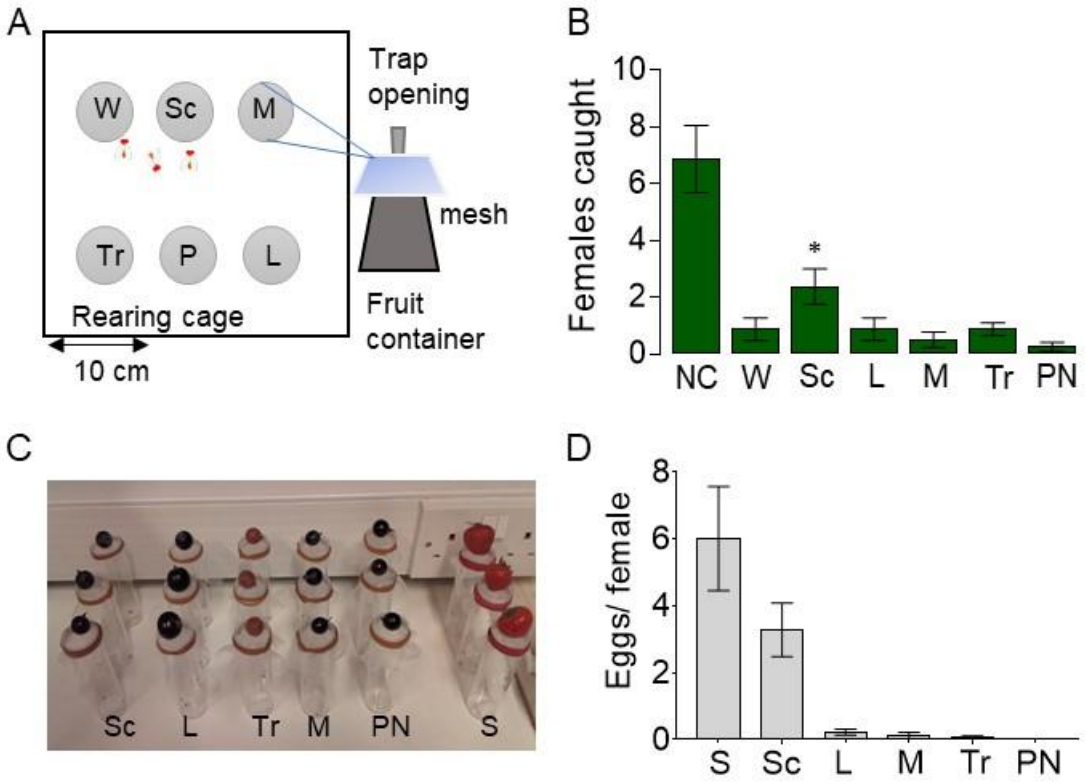


Figure 5-1 Olfactory discrimination of hosts and non-suitable wine grape cultivars for oviposition

A) Multiple choice assay. Fruits were hidden in flasks and flies were able to enter via a narrowed entry on the top. Headspaces reached out from the top of the flasks through a mesh. B) mean ± SEM number of females caught in each bait and in the cage, after 24h. *Significant difference between baits Schiava and water [Friedman test, F=12.68, P= 0.026]. N=8 with 10-20 females. C) Oviposition assays. D) mean ± SEM number of eggs laid per female for 24h. N=9 with three mating pairs. Baits were water (W), Strawberry (S) and five wine grape cultivars: Schiava (Sc), Lagrein (L), Merlot (M), Traminer (Tr) and Pinot Noir (PN). Females left in the cage were collected as no choice (NC).

However, most females did not choose any of the baits offered with only 45% of the flies trapped after 24 h. This suggests that the multiple choices assay was not optimized (Appendix 1). Additional behavioural tests are therefore necessary to characterise the behavioural response of gravid females to several cultivars of grape.

5.3 *DETECTION OF GRAPE CULTIVARS BY SEVEN FRUIT ACTIVATED OLFACTORY RECEPTOR NEURONS*

The next step was to determine whether the difference in attraction between the attractive cultivar Schiava and the non-attractive cultivars can be explained by differences in their

headspace composition. Then, fruit volatiles identified from these cultivars can be tested for electrophysiological responses by the seven classes of ORNs activated by ripe fruits (Chapter 3). This may help determining how the peripheral olfactory system in gravid females *D. suzukii* enable the discrimination of suitable oviposition substrates.

5.3.1 Headspace composition of four grape cultivars.

Using the same approach as in Chapter 4, grape headspaces were characterised and compared on their composition in bioactive compounds and their chemical class (Table 5-1). Headspaces were collected from the five wine grape cultivars used above. Antennally active GC-FID peaks were located from GC-EAG runs on adult female *D. suzukii*. The corresponding chemicals in all but the Pinot Noir extract were tentatively identified by GC-MS by John Caulfield (Rothamsted Research). The active headspace volatiles of two cultivars, Schiava and Traminer, were then identified with co-injection by Damien Lebouille and József Vuts (Rothamsted Research). Headspaces of Pinot noir, Merlot and Lagrein cultivars were not analysed due to time constraints. The composition must be considered preliminary and informative until the compounds identities are verified with co-injection and EAG (Method, 2.8).

The four grape cultivars released distinct headspace profiles (Table 5-1). The chemicals (*E*)-2-hexenal, 1-hexanol, benzaldehyde and isoamyl acetate were found in the headspace of both Schiava and at least one of the other cultivars. Unfortunately, no quantification allowed a comparative analysis of the amounts released for all cultivars.

There was no difference between Schiava (the susceptible cultivar) and the other cultivars in the proportions of chemicals from different classes and their composition do not appear to solely explain a difference in attractiveness. In order to determine which of these chemicals may be associated with the discrimination of the attractive cultivar, their response patterns on the peripheral olfactory system were determined.

Table 5-1 Antennally active compounds from headspaces of grape cultivars

Antennally active compounds identified from GC-EAG on antenna of gravid female *D. suzukii*. Tentative identification verified with GC-MS and co-injection on headspace extracts from Schiava and Traminer cultivars. Compounds were only tentatively identified (GC-MS only) from headspace extracts of Merlot and Lagrein cultivars. KI, Kovat Indices. CAS number indicated for the chemicals used for co-injection.

Compound	KI_{GC-EAG}	KI_{GC-MS}	Chemical class	CAS
Traminer				
(*E*)-2-Hexenal	828	827	Aldehyde	6728-26-3
1-Hexanol	855	855	Alcohol	111-27-3
Heptanal	886	880	Aldehyde	111-71-7
γ-Pentalactone	899	902	Ketone	108-29-2
1-Methylethyl ester-pentanoic acid	932	926	Acid	18362-97-5
6-Methyl-5-hepten-2-one	964	965	Ketone	110-93-0
2-(2-ethoxyethoxy) Ethanol	976	976	Alcohol	
Nonanal	1081	1083	Aldehyde	124-19-6
Schiava				
Ethyl cyclopentane	731	725	Cycloalkane	1640-89-7
Butyl acetate	762	759	Ester	123-86-4
2-Butanone	811	812	Ketone	78-93-3
(*E*)-2-Hexenal	828	828	Aldehyde	6728-26-3
1-Hexanol	855	855	Alcohol	111-27-3
Isoamyl acetate	862	862	Ester	123-92-2
Benzaldehyde	933	930	Aromatic aldehyde	100-52-7
4-Ethylstyrene	1079	1078	Cyclic alkene	3454-07-7
Merlot				
(*E*) or (*Z*)-2-Heptene	704	702	Alkene	
2-Ethyl-3-methyl-1-pentene	746	746	Alkene	
Isobutyl acetate	762	758	Ester	
2,3 or 1,3-Butanediol	771	770	Alcohol	
3-Ethyl cyclohexane	804	802	Cycloalkane	
Isoamyl acetate	861	862	Ester	
2-Methylbutyl acetate	863	865	Ester	
Heptanal	876	876	Aldehyde	
Isopropyl pentanoate	919	926	Ester	
Benzyl alcohol	999	1004	Aromatic alcohol	
2-Ethyl hexanol	1005	1015	Alcohol	

2-Butenyl benzene	1077	1078	Aromatic
Nonanal	1084	1084	Aldehyde
Benzaldehyde	1177	1182	Aromatic aldehyde
Lagrein			
2-Methylpropanoic acid	746	746	Acid
1,3-Butanediol	757	756	Alcohol
Hexanal	778	777	Aldehyde
3-Methylbutanoic acid	827	831	Acid
1-Hexanol	854	855	Alcohol
3-Methylbutanal acetate	861	862	Ester
4-Pentalactone	899	902	Ketone
Benzaldehyde	924	931	Aromatic aldehyde
6-Methyl-5-hepten-2-one	964	965	Ketone
2-(2-ethoxyethoxy) Ethanol	976	977	Alcohol
Nonanal	1084	1083	Aldehyde
Phenylethyl alcohol	1088	1086	Alcohol
m-Ethylacetophenone	1230	1236	Aromatic ketone
Tridecane	1292	1301	Alkane
Dodecanal	1444	1458	Aldehyde
Isopropyl dodecanoate	1610	1612	Ketone

5.3.2 Detection of grape headspace volatiles by fruit activated ORNs

The next step of the study was to identify which ORNs responded to the different grape cultivars. The aim was to identify the patterns of activation of each component to determine which are likely to induce attraction to the gape cultivar Schiava. These would activate at least two of the host activated ORNs: pb1A, ab1A, ab2A and ab3A (Chapter 3). Activation above 50 impulses/s are considered the most relevant to induce a behavioural response to fruit odours following results from Chapter 3 and Chapter 4. These appeared to be the most activated which would enable flies to recognise fruits from a background of odours and from diluted odour plumes under field condition, as discussed Chapter 3.

It was not possible to record the responses of ORNs to whole fruit headspaces thus, fruitprints for each cultivar could not be characterised. It was therefore not possible to identify which classes of ORNs were specifically activated by the susceptible and non-susceptible grape cultivar headspaces.

However, the identified compounds were tested on the seven classes of ORNs enabling the identification of potentially attractive and unattractive volatiles. Compounds from the Merlot

and Lagrein cultivars were only tentatively identified hence, were not tested with SSR. The affinity of Schiava and Traminer bioactive compounds was tested on the seven classes of ORNs activated by fruit headspaces. Responses to a stimulus made of 30 µl of a 0.01% solution (Chapter 4) were recorded. Occurrence of chemicals in other grape cultivars was also reported for a tentative association of activated ORNs and non-attractiveness of the cultivar (Figure 5-2).

Schiava grapes released 2-butanone, butyl acetate and isoamyl acetate. These chemicals induced a strong activation (> 50 impulses/s) from the classes of ORNs pb1A, ab1A, ab2A and ab3A, associated with attraction (Chapter 3). The unattractive cultivars shared volatiles which activated classes of ORNs associated with non-attraction, notably via activation of ab7A: 6-methyl-5-hepten-2-one and 1-hexanol. The first was not identified in Schiava.

The class ab4A was the most activated by all grape cultivars. (*E*)-2-hexenal was the only ligand for ab4A found in seemingly similar amounts in both cultivars from Schiava and Traminer (comparison based on the size of the GC-FID peak from which the chemical was identified) and may therefore not explain the difference in attractiveness observed.

Many compounds induce a similar activation of pb1A. Chemicals were all tested as part of a same experiment on pb1A, except for chemicals identified from other fruit types (Chapter 4). Notably, the response to benzaldehyde is higher than expected as higher doses did not induce an activation (Chapter 3, Figure 3-1). No contamination was identified but the experiment should be replicated to ensure the response rates to the chemicals only are recorded.

It can be concluded that both the activation of ab1A, pb1A, ab2A and ab3A and non-activation of ab7A may render Schiava more attractive to gravid females *D. suzukii*, than other cultivars. However, only the whole fruitprint given by the whole headspace will enable to conclude on which ORN activation pattern drove the different behaviours towards grape cultivars. The chemicals 2-butanone, butyl acetate, isoamyl acetate and 6-methyl-5-hepten-2-one appear the most associated with these cultivar specific activations.

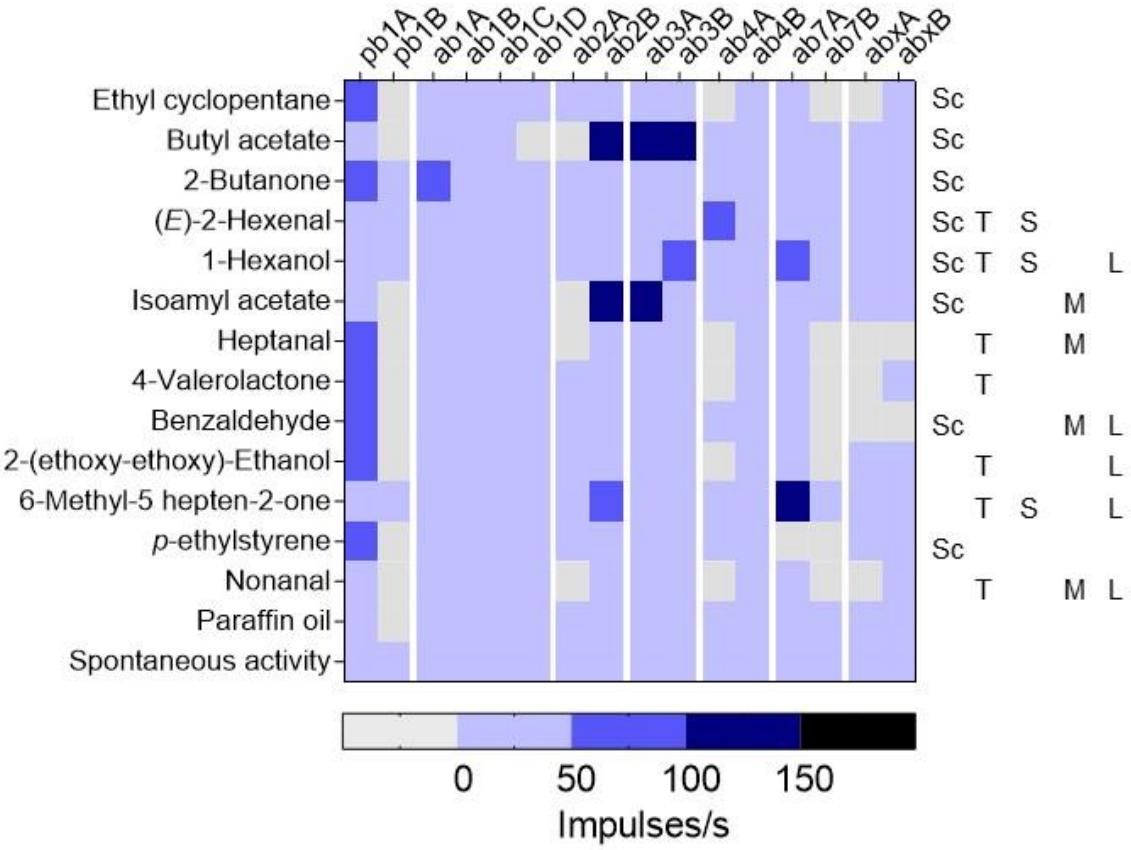


Figure 5-2 Activation of classes of ORNs by grape headspace volatiles

Response rate during a 0.5 s stimulus with 30 µL of a 0.01% solution in paraffin oil, showing an increase (light to dark blue) or decrease (white) compared to the spontaneous activity. Volatiles were characterised from headspaces extracts of grape cultivars Schiava (Sc), Traminer (T), Sugraone (S), Merlot (M) and Lagrein (L). Data for Sugraone taken from chapter 4. The identification of isoamyl acetate in Schiava extract. And of compounds in Merlot and Lagrein extracts was not completed. Compounds are ranked from the highest to the lowest volatility (Kovat Index from the GC-EAG identification).

5.4 DISCUSSION

5.4.1 Discrimination of cultivar-specific headspaces by *D. suzukii*

Can female *D. suzukii* discriminate among cultivars of the same plant species because of differences in intensity and ratio of activation of ORNs forming the fruitprints? Or do they detect all cultivars similarly and determine their suitability for egg laying solely upon landing? Variation in infestation were noted in grape cultivars in different regions of the world. Vineyards are not all concerned by *D. suzukii* because not all cultivars have been damaged in comparison to other small fruit and berry orchards (Bellamy *et al.* 2013; Ioriatti *et al.* 2015; Lee *et al.* 2015). However, significant damages on commercially important cultivars such as Schiava or Chardonnay urge for protection methods (Ioriatti *et al*. 2015).

The present study demonstrated that grape cultivars are discriminated by their headspace volatiles, suggesting that the defended hypothesis is true: gravid females *D. suzukii* can discriminate cultivars with suitable fruits for oviposition, of a same plant species via cultivar specific activation of classes of ORNs on their peripheral olfactory system. Indeed, females displayed an increased attraction for one of the cultivars when only headspaces could be used as a cue. Furthermore, the chemical composition of headspaces showed that each cultivar released distinct volatile bouquet that are detected. Cultivar can indeed release very distinct aroma profiles (Vilanova *et al.* 2007). The fruitprints could not be seen, but the subsets of ORNs activated by these volatiles indicate that a difference in attractiveness is associated with the activation pattern of ORNs as discussed chapter 4. Candidate volatiles and candidate ORNs were identified as likely involved in the attractiveness of the Schiava grape. A model of olfactory discrimination in which dedicated classes of ORNs are activated in combinatorial patterns described in chapter 3 and chapter 4 is supported by this study. This study was however preliminary due to a poor behavioural response rate. Furthermore, time and logistic constraints prevented a full chemical and electrophysiological study of the headspaces. It thus requires additional experiments.

5.4.2 Discrimination of grape cultivars: roles of pb1A, ab1A, ab4A and ab7A

Ligands for ab1A, pb1A and ab2B were identified in many of these grape cultivars, but particularly, the Schiava had a larger amount of them, in addition to low amount of ab7A activating compounds (see above). Both the activation of ab7A and reduced activation of ab1A and b1A may be the reason why only the Schiava cultivar is attractive among all grape cultivars tested. It therefore confirmed that host discrimination involves at least two combinations of ORNs: The host activated pb1A and ab1A, and the non-host activated

ab7A and ab4A. As discussed in previous chapters, combinations of host activated and non-host activated ORNs may be simultaneously activated and, depending on their numbers and intensity may induce different degrees of attraction. Crosstalks and interactions between glomeruli and ORNs may additionally shape the fruit-specific responses from fruitprints (van der Goes van Naters 2013; Mohamed *et al.* 2019).

5.4.2.1 The activation of pb1A and ab1A induce attraction to Schiava cultivar

Based on the composition of headspaces pb1A and ab1A were activated by Schiava headspaces only. These ORNs may be involved in the discrimination of Schiava as an attractive cultivar, supporting a role of pb1A and ab1A in mediating attraction to host fruits (chapter 3 and 4). The activation of pb1A by chemicals identified from grape cultivars must be carefully considered. All odours activated ORNs similarly while being tested in a same experiment. Even though controls (paraffin oil) and a troubleshoot did not identify any source of contamination data should be reproduced to verify whether these chemicals are ligands for pb1A.

5.4.2.2 The ab7A prevents attraction to non-susceptible cultivars

The ab7A may be activated with higher intensity by the headspaces of unattractive cultivars as more ligands for ab7A were produced. These results support findings from other chapters of this thesis demonstrating that ab7A seem to be involved in aversion (Chapter 3, 4 and 6). In earlier studies, the number of compounds and number of ORNs activated were associated with attraction to fruits (Dweck *et al*. 2016; 2018). However, the spectrum of activation of a chemical depends largely on the dose used and its affinity for ORNs. Furthermore, results from Chapter 3 and 4 demonstrate that whole headspaces did not activate the same ORNs than each of its components. Therefore, quantifications of fruit compounds would be required along with fruitprints to conclude on how the olfactory representation of headspaces drive the behavioural responses to grape cultivars.

5.4.2.3 The ab4A associated with non-hosts

The only ligand for ab4A identified in grape headspaces was (*E*)-2-Hexenal. The ab4A appears associated with non-attractiveness and with detection of ripeness (Chapter 3). At this stage, the activation of ab4A by (*E*)-2-hexenal cannot explain a difference in attractiveness among cultivars. Indeed, the amounts of this chemical appeared similar in attractive and non-attractive cultivars but without measure. The activation of ab4A was found specific to three grape cultivars and to tomato (chapter 4) and is seemingly associated with the activation of ab7A. All but the Schiava cultivar were not attractive to *D. suzukii* and not susceptible to egg laying. As discussed in chapter 4, ab4A may be associated with detection of non-suitable fruits.

5.4.3 Prospects for pest control: candidate semiochemicals

Grape cultivar-specific volatiles may be candidate for development of olfactory lures and disruption for pest management. The model from chapter 3 was validated here. By deciphering which ORNs are activated by headspace volatiles of attractive and unattractive grape cultivars, it is possible to identify candidate attractants or repellents. The study demonstrated that the candidate attractant and repellent (or masking cues) identified in Chapter 3 as involved in the discrimination of host fruits, are also involved in the discrimination of susceptible grape cultivars. Candidate attractant are ethyl cyclopentane, 2-butanone, isoamyl acetate and butyl acetate. Candidate masking cues /repellents are 6-methyl-5-hepten-2-one, 1-hexanol and (*E*)-2-hexenal.

5.5 CONCLUSION AND FUTURE DIRECTIONS

The results presented in this chapter show evidence that Schiava cultivar is recognised by gravid females *D. suzukii* as a host, possibly because the headspace bouquet strongly activated a combination of the pb1A and ab1A neurons and simultaneously did not activate a combination of the ab7A, ab4A and abXA, contrary to non-suitable grape cultivars headspaces. This separate study supports the results of chapter 3 and 4, that a combinatorial pattern of activation involving the pb1A, ab1A, ab4A and ab7A enable females to discriminate host and non-suitable fruits for oviposition. Cultivar of grapes released distinct chemical profiles which fly processed and discriminated with their peripheral olfactory system. It shows the extent with which the olfactory system enables a precise encoding of fruit odours to discriminate fruits from plant species that are taxonomically diverse and taxonomically closely related. Additional research is needed to complete the present study and fully characterise which specific olfactory circuitry and chemicals enables the fly to discriminate suitable grape cultivars for oviposition before landing on the fruit.

CHAPTER 6

6 FRUITPRINTS IN *D. MELANOGASTER*: A COMPARATIVE STUDY OF RIPENING FRUIT DETECTION

Drosophila suzukii and its reflected image

6 FRUITPRINTS IN *D. MELANOGASTER* AND FUNCTIONAL SPECIALISATION OF RIPE FRUIT DETECTION IN *D. SUZUKII*

6.1 INTRODUCTION

6.1.1 Aim of chapter

To gain insight into how the detection of host volatiles by olfactory receptor neurons (ORNs) on the peripheral olfactory system of *D. suzukii* may be associated with host shift from overripe to ripening fruits, gravid female *D. melanogaster* were used as a comparative model. The aim of the study was to identify functional differences between homologous ORNs between species, regarding ripe fruit detection. Codon based computational methods for detection of selection suggest the majority of *Or* genes are under purifying selection, but a small subset show the signatures of positive selection (see below). The hypothesis addressed in this chapter is that ripe fruit odours are encoded similarly by the peripheral olfactory system in the two species but some classes of ORNs functionally differ thereby allowing females *D. suzukii* to be more sensitive to ripening fruits that are suitable for oviposition.

Furthermore, it may be possible to identify repellent semiochemicals by testing their affinity for a number of specific ORNs. In both *D. suzukii* and *D. melanogaster*, the class ab10B respond to a racemic mixture of nepetalactol (identified from parasitoid wasp body odour, see below). Enantiospecificity exists among ORNs in *D. suzukii* (Chapter 4) therefore, the detection of nepetalactol in *D. suzukii* is a suitable model to test the following hypothesis: enantiomers and their racemic mixtures are encoded by different combination of ORNs, thereby mediating different behavioural responses. Only one of the enantiomers could be used in this research and results were compared to earlier studies.

First, 28 classes of ORNs housed in basiconic sensillum types were characterised in *D. melanogaster* using the same panel of chemicals used in *D. suzukii* (chapter 3) to ensure a correct identification of homologous ORNs. The headspaces of ripening whole fruits from the same plant species as used for *D. suzukii* (Chapter 3) were then tested on all basiconic classes of ORNs of *D. melanogaster.* The responses to ripe fruit odours and fruit volatiles in the two species were then compared in order to identify the dissimilarities involved in the specialisation to ripening fruits. The (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol, identified from repellent wasp body odour was tested on all classes of ORNs from the antenna in *D. suzukii* and *D. melanogaster*.

The results presented in this chapter bring new insights on how the two species differ in their encoding of ripening fruits and cues associated with suitability for oviposition and detection of danger by the peripheral olfactory system. The identification of a *D. suzukii*

specific olfactory circuitry mediating avoidance would permit to identify semiochemicals that are repellent. Also, the identification of inhibitors for the ORNs mediating avoidance may be used as novel tools to increase the success of biocontrol via natural enemies, particularly with parasitoid wasps.

6.1.2 Background

Host shifts and specialization is associated with changes in olfactory detection. Selection and loss of sensory units are dependent on the requirements of the novel ecological niche (Hansson and Stensmyr 2011). The host shift from overripe to ripening fruits has not yet been unravelled, but a few studies have underlined the role of olfaction, and the differences in the detection of fermenting products between *D. suzukii* and *D. melanogaster* (Keesey *et al.* 2015, Scheidler *et al.* 2015).

6.1.2.1 The olfactory system of Drosophilids

The peripheral olfactory system was described in *D. melanogaster* and homologous structures were named similarly in other Drosophilids. Olfactory receptor neurons (ORNs) are housed in peg-like or hair-like structures on the surface of the antenna and maxillary palps. Functional structures called basiconic sensilla house two to four ORNs tuned to food/host odours (Introduction, 1.3). ORNs are associated with olfactory receptors (ORs) which are tuned to a few or many different food and host volatiles. Approximately 13 functional types of basiconic sensilla have been identified, labelled ab1 through ab10 on the third antennal segment and pb1, pb2 and pb3 on the maxillary palp. The ORNs can be recognise by the amplitude of their action potentials on electrographs and are labelled according to this amplitude and to the housing sensillum type (Method, 2.8.2). For instance, the basiconic sensillum type ab1 house four ORNs: ab1A, ab1B, ab1C and ab1D. These ORNs once activated, relay sensory signals towards centralised olfactory units (Introduction, 1.3).

Extracellular recordings (SSR) of the activity of these ORNs allows their response to chemical stimuli to be measured (Kaissling 1995). 13 Basiconic types housing 28 classes of ORNs have been characterised in *D. suzukii* in this study (Chapter 3) and by Keesey *et al.* (2019). Their responses to ripe fruit odours that are host and non-host have been measured, identifying seven ORNs dedicated to ripe fruit detection (Chapter 3). The functional type ab9 was however not identified by homology to the one described in *D. melanogaster* in earlier studies and an additional novel type abX was discovered. The abX was identified and considered to be the ab9 by Keesey *et al*. (2019).

6.1.2.2 Evolution of the olfactory system

Death-birth evolutionary events of selected genes of the OR repertoire along the various existing lineages, supports the ecological host shift of many Drosophilids (Dekker *et al.* 2006; Ometto *et al.* 2013; Hickner *et al.* 2016; Ramasamy *et al.* 2016). Evolutionary changes of the chemosensory repertoire have been associated with host specialisation in (among others) *D. sechellia*, *D. erecta* and Hawaian *Drosophila spp.*, from yeast feeding to their respective specialised host plants (Dekker *et al.* 2006; McBride 2007; McBride *et al.* 2007; Goldman-Huertas *et al.* 2015). These events are thought to be either a drive or a consequence of host shifts and were explored in *D. suzukii* by Hickner and colleagues (2016) and Ramasamy and colleagues (2016) and summarised below (Table 1-1).

Another example is a host race based on fruit odour discrimination in *Rhagoletis* flies (Linn *et al.* 2003; Forbes and Feder 2006; Tait *et al.* 2016). A host shift was led by olfactory switch in the fruit fly *Rhagoletis pomonella*, in which four populations have diverging host preferences: the ORN classes responding to fruit volatiles had been characterized in these populations leading to the discovery of a reversal in the response to two key attractive volatiles by two ORN classes. The switch of response in two populations led to an adaptation on two different hosts (Tait *et al.* 2016).

Phylogenetic studies on 10 closely related *Drosophilidae* from the *melanogaster* group revealed that most of the orthologous chemosensory genes encoding ORs, GRs, IRs and OBPs, are highly conserved across species. In addition, electron microscopy images revealed anatomical similarities between *D. suzukii* and *D. melanogaster* (Hickner *et al.* 2016). Two major evolutionary events were found in the lineage to *D. suzukii*. The repertoires of OR genes *Or74a*, *Or85a, and Or98b* were lost, or changed function compared to the orthologues found in other lineages (Hickner *et al.* 2016; Ramasamy *et al.* 2016). Particularly, Or74a was conserved in all but *D. suzukii*. Ramasamy and colleagues further annotated the loss of *Or98a*. These *Or* genes were expressed in *D. melanogaster* larva (unknow ORN), ab2B, and ab6B ORNs respectively, and *Or98a* is expressed in ab7A.

6.1.2.3 Evolutionary event: expanded repertoires

Two lineages underwent large expansions, with four copies of the *Or23a* and *Or67a* genes, and one *Or67a* pseudogene. The latter was also found by Revadi and colleagues (2015). These two are expressed in ai2C and ab10A respectively. Expansion, or duplication of *Or42a*, splice variants of *Or46a* and duplicated splice variants of *Or69a* were also reported in *D. suzukii* and its close related *D. takahashii*. All genes which underwent an evolutionary event were orthologues of the *D. melanogaster* genes, coding for ORs with high affinity to esters and yeast/ fermentation derived compounds, hereby providing a link between evolutionary changes of ORs and detection of host compounds (Ramasamy *et al.* 2016).

This is the case for pb1A (with Or42a) responding to attractive fruits in *D. suzukii* (Chapter 3). However, the roles of these ORNs are not all understood as for instance the ab10A, co-localised with the danger detecting ab10B (Ebrahim *et al.* 2015). The roles of pb2B (with Or46aA) and ab9A, ab9B (with Or69a) remain also unclear.

6.1.2.4 The role of Or22a and Or22b

Ramasamy and colleagues (2016) further identified in the lineage of *D. suzukii* and *D. biarmipes*, a loss of function for *Or22a* and a duplication of *Or22b* (both expressed with ab3A). A change in function of *Or22a* and *Or85a* (expressed in ab2B) was additionally suggested instead of a pseudogenisation. Hickner and colleagues (2016) did not find changes on these two genes possibly because of the different technique used and a stricter correction of their statistical analyses, which reduced their number of significant candidates compared to earlier phylogenetic studies. Furthermore, two different techniques on the genomes of two strains of *D. suzukii* and *D. biarmipes* (the closest related species) were used hereby increasing the detection of strains variability (Ometto *et al.* 2013; Ramasamy *et al.* 2016). The orthologues of the gene *Or22a* (expressed in the ab3A ORN as described in *D. melanogaster*), were found involved in host shifts in four *Drosophila spp.* (see above), including a change of sensitivity of the ab3A ORN to fermenting odours in favour to ripening fruit related odours in *D. suzukii*, implying its association with Or22a as in *D. melanogaster* (Schlyter *et al.* 2012; Keesey *et al.* 2015; Ramasamy *et al.* 2016). Further characterisations of the functional divergences of these ORs in *Drosophila* would be valuable in addition to these phylogenetic studies.

6.1.2.5 Enhanced detection of the ripe fruit volatile isoamyl acetate

The detection of the fruit volatile isoamyl acetate was highlighted by Revadi and colleagues (2015). Isoamyl acetate is a component of the bouquet of various ripening, ripe and early fermenting fruits. It was consistently present and active at low concentration in ripe fruit extracts (hosts for *D. suzukii*) tested with GC-EAG (Abraham *et al.* 2015; Revadi *et al.* 2015). A repertoire of 10 orthologue genes coding for ORs responding to isoamyl acetate in *D. melanogaster* were identified and annotated in *D. suzukii* (Ometto *et al.* 2013; Hickner *et al.* 2016; Münch and Galizia 2016).

The expanded *Or67a* gene is expressed on the membrane of ab10A ORNs (Hallem and Carlson 2006). Its affinity to isoamyl acetate which was found attractive, also highlight the potential role of ab10A and Or67a in ripe fruit detection (Revadi *et al.* 2015). This further indicate that ORNs of the ab10 sensilla may be involved in two separate mechanisms: host detection by the ab10A (Revadi *et al.* 2015), and detection and avoidance of the *Leptopilina sp.* parasitoid wasp by its co-localised ab10B (Ebrahim *et al.* 2015).

6.1.2.6 Exploiting natural enemies for control of D. suzukii

Biological control using parasitoid wasps that are natural enemies for *D. suzukii,* were mentioned as early as 1939 by Kanzawa. The technique remained limited because of the stronger immune system of *D. suzukii* larvae, compared to other Drosophilids, in encapsulating the larval parasitoid, even from the most successful *Asobara japonica* Belokobylskij (Daane *et al.* 2016), *Pachycrepoideus vindemiae* Rondani, *Leptopilina heterotoma* Thomson and *Trichopria drosophilae* Perkins (Mitsui and Kimura 2010; Chabert *et al.* 2012; Kruitwagen Astrid *et al.* 2018). Natural populations of parasitoid wasp species are being monitored alongside with *D. suzukii* and other drosophilids to determine their potential as a biological control. *T. drosophilae* is being released in experimental trials in cherry orchards in Italy (Pers. comm., V. Rossi and A. Grassi, FEM) (Rossi Stacconi *et al.* 2013; Rossi Stacconi *et al.* 2015).

The ORN class ab10B mediates avoidance via the detection of adversive compounds from *Leptopilina spp.*, in both *D. melanogaster* and *D. suzukii* (Ebrahim *et al*. 2015). One of the wasp body odour compounds which was antennally active is nepetalactol, an iridoid showing insect repellency (Zhu *et al.* 2011). However, the exact enantiomer(s) was (were) not identified. A thorough identification of the enantiomers constituting the wasp body odour may enable to isolate semiochemicals to be used in biocontrol (Chapter 4, 4.1.2.1).

6.2 CHARACTERISATION OF FRUITPRINTS ON THE PERIPHERAL OLFACTORY SYSTEM IN D. MELANOGASTER

6.2.1 Thirteen functional basiconic sensillum types

Thirteen functional sensillum types were characterised by their location and by the response of their associated ORNs to diagnostic panels of chemicals in *D. melanogaster* in earlier studies (Introduction, 1-3) and the same chemicals were used for *D. suzukii* (Chapter 3). All but the ab9 and abY were identified from previous work (Introduction, 1,3). The types ab1-ab8, ab10 and aby were recognised on the antenna. The types pb1-pb3 were identified on the maxillary palps by W. van der Goes van Naters using a different panel of ligands. The ab1 housed four ORNs (A-D) while all others housed 2 ORNs (A and B).

For each sensillum type at least one key chemical was identified. Key chemicals allowed immediate functional identification of a sensillum type because one of the ORNs was particularly sensitive to it and this ligand was not equally active on other sensilla. These key ligands are CO_2 for ab1C, ethyl acetate for ab2A, 2-heptanone for ab3B, (*E*)-2-hexenal for ab4A, pentyl acetate for ab5B, (*RS*)-1-octen-3-ol for ab6A, ethyl lactate for ab7A, ethyl butanoate for ab8A, 2-phenyl ethanol for ab10A, and ethyl benzoate for abY. Responses to these key ligands were measured across all sensillum types (Figure 3-1), as were

responses to other chemicals used (Appendix 9). 6-methyl-5 hepten-2-one is another key ligand for the ab7A.

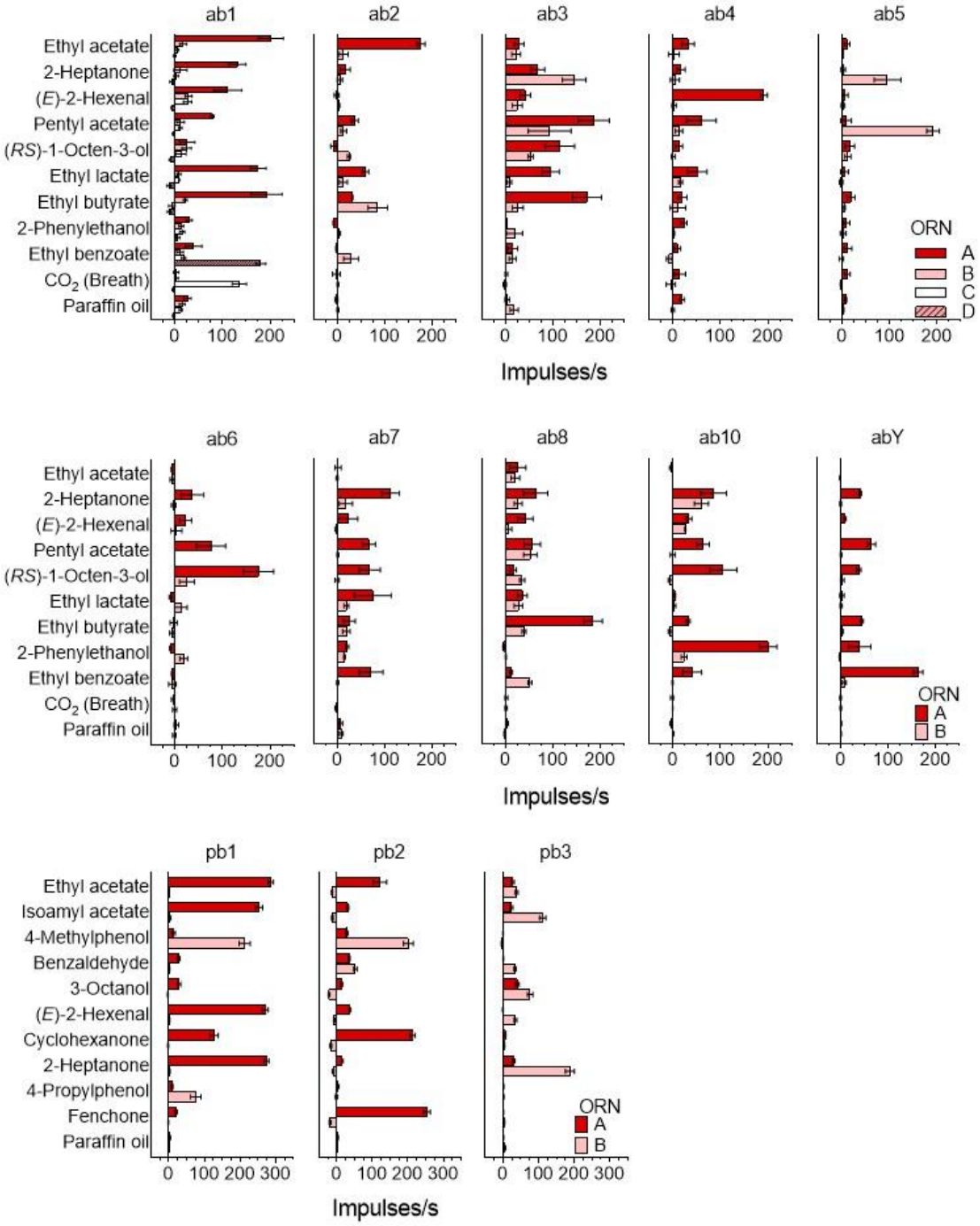


Figure 6-1 Antenna and palp basiconic olfactory receptor neurons (ORNs) in D. melanogaster

Sensillum type on the antenna: ab1,…, abX and on the maxillary palp: pb1, pb2 and pb3. ORNs are labelled A, B, C or D by the decreasing amplitude of their fired action potentials (impulses). Mean ± SEM impulse rate during a 0.5 s stimulus. Stimuli consisted of an air pulse through a glass cartridge containing 30 µl of a 1% solution in paraffin oil. Unless stated otherwise, chemicals were racemic and > 95% pure. The CO_2 stimulus was a

glass cartridge filled with exhaled air. The spontaneous activity was subtracted from all responses. N=3-6 from at least three females.

6.2.2 Fruitprints on the peripheral olfactory system of *D. melanogaster*

In *D. melanogaster*, fruitprints or pattern of activation from fruit headspaces on the classes of ORNs of the peripheral olfactory system were determined using ripe fruit headspaces. These fruits were characterised as host and non-host for *D. suzukii*. These ripe fruits were harvested and used as whole, with undamaged skin. It is hypothesised that none of them are hosts for *D. melanogaster* as their flesh was not accessible to larvae (Atallah *et al.* 2014).

The strawberry fruitprint can be characterised with the highest increase in response rate compared to the spontaneous activity of the classes ab1A, ab2A, ab3A (> 100 impulses/s) and the classes pb1A and ab4A (approximately 50 impulses/s). Raspberry fruitprint can be characterised by ab1A (> 150 impulses/s), ab2A, pb1A (> 100 impulses/s) and ab3A (about 50 impulses/s). Blueberry headspaces activated only ab1A (approximately 100 impulses/s). Orange headspaces are characterised by an increased activity of ab3A (> 100 impulses/s) ab1A, ab7A and abYA (approximately 50 impulses/s). Grape headspaces activated ab4A (> 100 impulses/s), ab1A and ab3A (approximately 50 impulses/s). Lastly, tomato headspaces activated ab7A (>100 impulses/s) and ab4A (> 50 impulses/s).

Overall, these fruitprints are specific to each fruit type. It can be concluded that the peripheral olfactory system in *D. melanogaster* enables discrimination among fruit from taxonomically diverse plant species.

In order to determine which of these activations were the strongest and which ORNs were not activated a hierarchical cluster analysis (HCA) and statistical analysis of variance were used. The HCA permitted to distinct two clusters of ORNs which grouped by the similarity of their responses to fruit headspaces. As discussed in Chapter 3, the higher firing rates may suggest that these ORNs enable to detect headspaces through a background of odours and may thus be more relevant than other activated ORN classes. Five classes: ab1A, ab2A, ab3A, ab4A and ab7A clustered together as the most responsive to ripe fruit headspaces. It was concluded that these classes are ripe fruit activated ORNs and are likely involved in any behavioural responses associated with the fruitprints as demonstrated for *D. suzukii* (Chapter 3). Next, Wilcoxon signed ranked tests showed that the classes ab2B, ab4B, ab5B, ab6B, ab10A and B, pb2A and B, and pb3B were not activated by fruit odours (not different from control air) and were therefore considered as non-fruit responding ORNs.

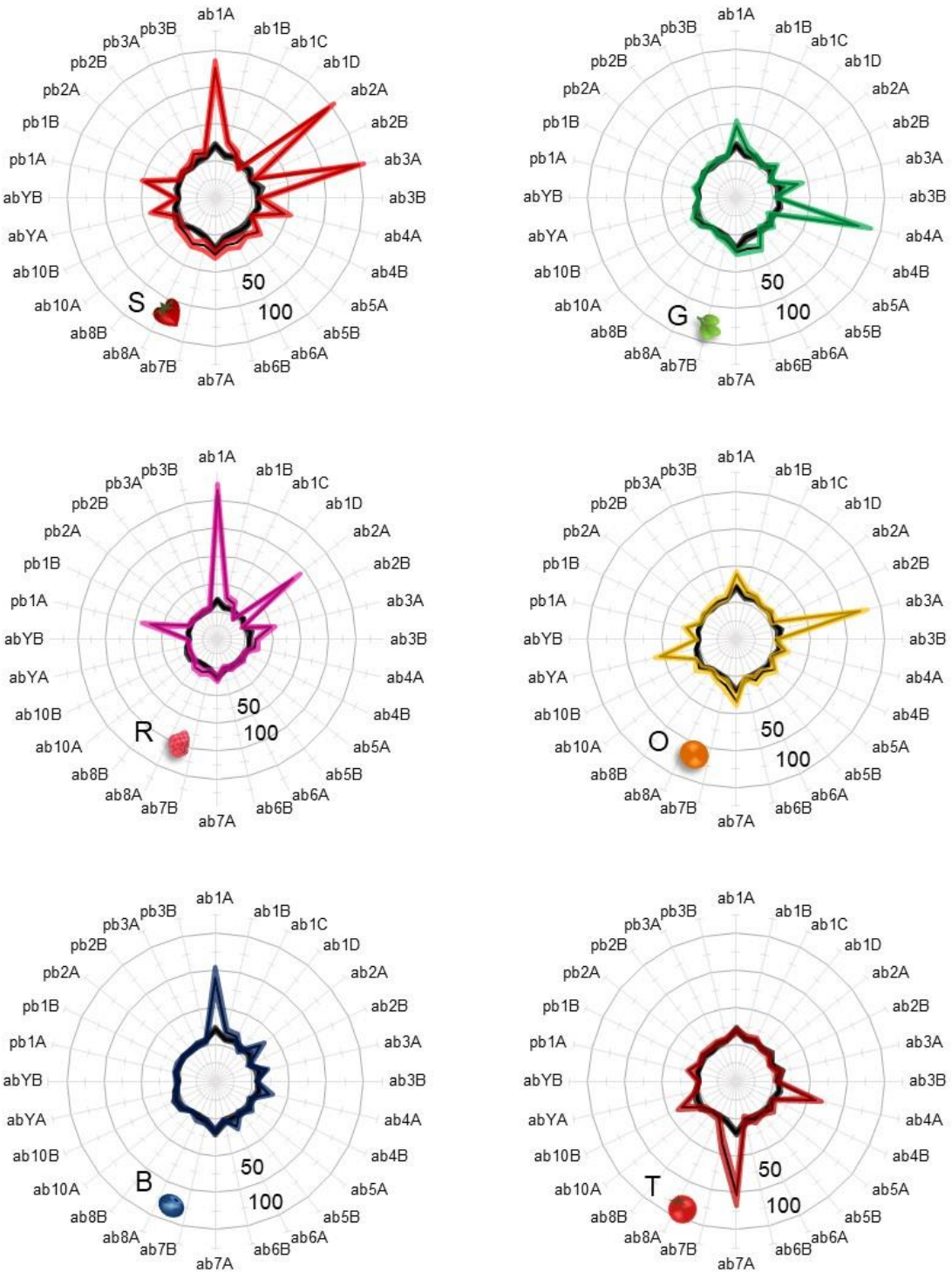


Figure 6-2 Fruitprints on the peripheral olfactory system of D. melanogaster

Mean response rate ± SEM (impulses/s) to a 0.5 s stimulus of whole harvested ripe fruit headspace by the olfactory receptor neuron classes of the antenna (ab1A-abXB) and maxillary palps (pb1A-pb3B). Strawberry (S, red), raspberry (R, magenta), blueberry (B, blue), grape (G, green), tomato (T, dark red) and orange (O, orange). Back line shows the mean response, coloured lines show the SEM range. The response to ambient air (grey) is shown in each graph. Axis range from -50 (center) to 150 impulses/s (periphery). N= 5-13.

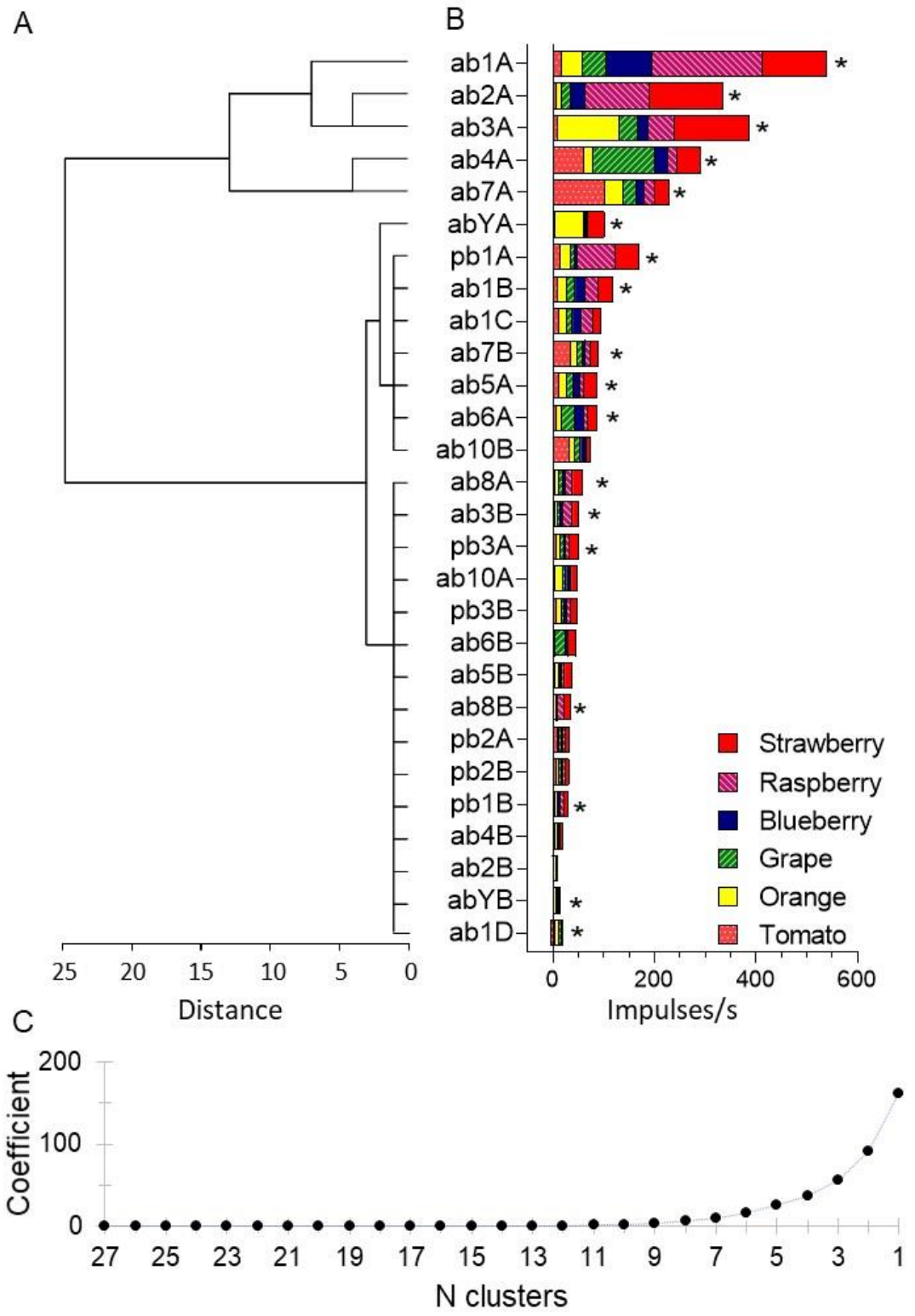


Figure 6-3 Classification of ORN responses to ripe fruit headspaces in D. melanogaster

A) Agglomerative Hierarchical Cluster Analysis using Ward's method on squared Euclidian distances. B) Stacked mean response rate to fruit headspaces for each ORN class. *Significant difference of at least one response to fruit headspace in comparison to control (stimuli with ambient air) using a Wilcoxon signed-ranked tests (Appendix 9). C) Agglomeration schedule of the hierarchical cluster analysis suggesting a two-clusters

solution: The distance coefficient between number of clusters is the largest between one and two clusters.

6.3 *FUNCTIONAL DIVERGENCE IN THE D. SUZUKII LINEAGE*

Given the striking similarity between species of the fruitprints are patterns of activation of ORNs in response to ripe fruit odours, a comparative study was done. The aim is to identify which of these Classes of ORNs underwent functional changes leading to a host shift from Overripe to ripening fruits. Data for *D. suzukii* were mined from chapter 3 and compared with data for *D. melanogaster* (see above).

6.3.1 Differences in spontaneous activity

Electrophysiological traces were similar between species as the impulses fired by the co-localised ORNs were of similar amplitude differences. One exception was noted for ab1A. the ab1A was not the ORN with the action potentials of largest amplitude as described by the nomenclature adopted for all other sensillum types (“A” largest amplitude, “B” second largest amplitude, etc.). The ab1A in *D. suzukii* had impulses of lower amplitude than ab1B. However, because ab1A had consistent similar responses to the diagnostic odour panel compared to ab1A in *D. melanogaster*, it was labelled accordingly (chapter 3).

Overall, the spontaneous activity was similar for homologous ORNs between the two species, with few exceptions for which a statistical analysis revealed significant differences. (appendix 9). The classes of ORNs ab1B, ab2A, ab2B, ab3A, ab10A, pb1B, pb2A and pb2B had a significant higher firing rate in *D. suzukii* compared to *D. melanogaster.*

It can be concluded that functional differences between the two species are present without stimuli. They are particularly observed for the most activated ORNs by headspaces from fruit hosts for *D. suzukii*, and/or their co-localised ORNs. It is unknown whether the spontaneous activity of ORNs is an existing physiological response or is produced as an artefact of recordings. Piercing the sensillum wall with the electrode may induce a change in potential in the sensillum lymph in which the ORN dendrites bath creating a spontaneous activity.

6.3.2 The ab9 and two novel functional types abX and abY

The type ab9 was not found in *D. melanogaster*, and either in *D. suzukii* (chapter 3). A literature review permitted to describe the two novel types in a comparison with the unidentified ab9 (Table 6-1). None of the ORNs recorded in this study responded to the chemicals of the panel as described for a said ab9. *Or69a* and *Or67b* orthologues are found and conserved in *D. suzukii*. It is possible that the panels used did not contain any ligands

of specific high affinity for ab9, rendering its identification difficult. It was concluded that additional recordings on the antenna are necessary using a different set of ligands, in order to identify ab9 in *D. melanogaster* and in *D. suzukii*, as this functional type is likely to be present on the antenna and may have been missed in this study.

An additional functional type abY was described. The responses of its two housed ORNs to the panel of odours, was not similar to the described functional types ab9 (see above) and the novel described abX in *D. suzukii* (Table 6-1). Both types housed two ORNs which action potentials were distinct and easily identified (Figure 6-4). Considered non homologous, the ORNs in the functional basiconic types abX and abY were therefore not compared to each other in the next sections of this study.

Table 6-1 Comparison of the functional types abX from Drosophila suzukii and abY from Drosophila melanogaster with ab9 ORs in response to chemicals.

Response rates (impulses/s), to stimuli with 30 µl of 1% solution in paraffin oil: "++++", n≥200; "+++", 150≤n<200; "++", 100≤n<150; "+", 50≤n<100; ".", n<50. Nerol was reported as ligand by Kreher *et al.* (2005), no numbers were retrieved. Data originated from Grabe *et al.* (2016), Kreher *et al.* (2005, 2008), Muench and Galizia (2016), Dweck *et al.* (2015), Lebreton *et al.,* (2017).

	DoOR index*		ΔF/F**	Impulses/s**		Impulses/s***			
	Or67b	Or69a	Or69a	Or67b	Or69a	abYA	abYB	abXA	abXB
1-Hexanol	0.741			++++		.	.	+	.
(*RS*)-1-Octen-3-ol	0.197			+		.	.	++	.
Acetophenone	0.943			++++[1]		.	.	.	.
Ethyl acetate	0.078			.		.	.	.	.
Pentyl acetate	0.366			++		+	.	+	.
Ethyl butyrate	0.017			.		.	.	.	.
Methyl salicylate	0.027	0.545	1.04	.		.	.	.	.
2-Heptanone	0.547	0.347	0.39	+++		.	.	+	.
2-Phenylethanol	0.832	0.471	0.8	++		.	.	.	.
α-Terpineol	0.044	0.764	1.75	+		.	.	.	.
(*R*)-Linalool	0.01	0.716	1.6		+++[2]	.	.	+++	.
(*E*)-2-Hexenal	0.427	0.277	0.16	++		.	.	.	.
Benzaldehyde	0.695	0.146	-0.26	++++		.	.	.	.
Geranyl acetate	0.05	0.38	0.5	.		.	.	.	.
Ethyl benzoate	0.377	0.455	0.75			+++	.	+	.
Isoamyl acetate		0.437	0.69	.		+	.	+	.
Sulcatone						+	.	++	.
Nerol					++++	.	.	+++	.

*DoOR index ranges from 0 (weakest ligand) to 1 (best ligand, the highest intensity of response) (Galizia *et al*. 2016). **Or69a and Or67b were ectopically expressed in empty neuron (Gal4-UAS constructs) and recorded using SSR and calcium imaging by Kreher *et al* (2005, 2008). Intensity of responses from Calcium imaging are reported as the mean

ΔF/F values ± SEM. ***abY (A&B) and abX (A&B) were recorded from *D. melanogaster* (described above) and *D. suzukii* (chapter 3) respectively, using extracellular SSR on wild-type gravid females. [1]Conflicting responses: n<100 by Kreher *et al.* (2008), n> 150 by Lebreton *et al.* (2017).[2]Conflicting responded: n= 50 by Dweck *et al.* (2015), n> 200 by kreher *et al.* (2005).

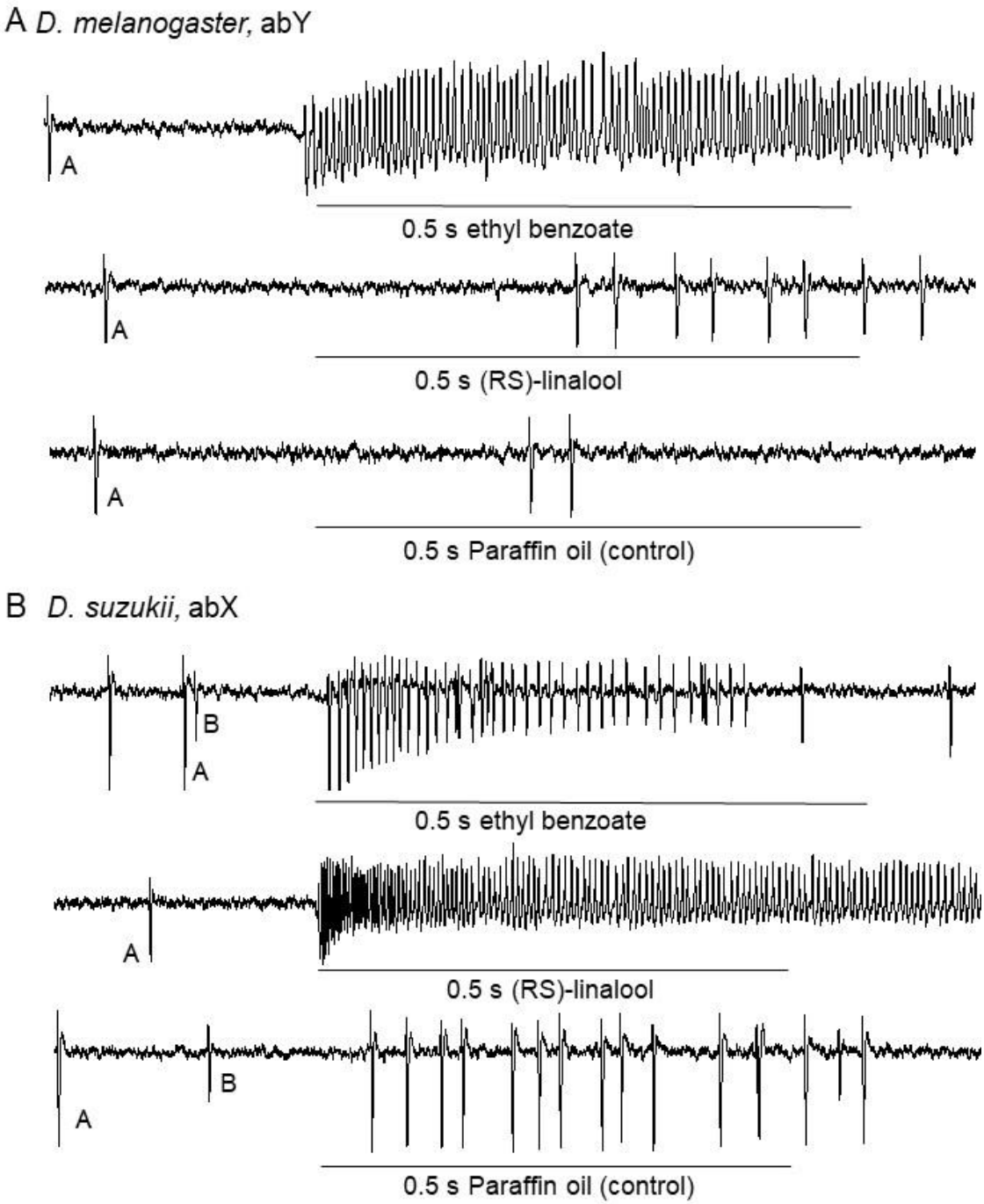


Figure 6-4 Traces of the abY and abX ORNs responses to chemicals

Response rate during a 0.5 s stimulus with 1% dose of ethyl benzoate, (*RS*)-linalool and paraffin oil (solvent). A and B annotated the action potentials of the two colocalised ORNs abXA and abXB, and abYA and abYB.

6.3.3 Ripe fruit detection

In order to determine whether some classes of ORNs may have changed in order to adapt (or as a result of adaptation) to a novel ecological niche, homologies between the characterised classes of ORNs was verified. The coordinates of responses for each species were calculated in a multidimensional space formed by the responses to all fruit headspaces tested. The Euclidian distance was thus calculated between ORNs in an orthogonal system. To determine which fruits were differently detected, a Wilcoxon signed-ranked test between the responses of the homologous ORNs was then performed to compare the two species (Method, 2.8).

The two species significantly differed by their responses to ripe fruit odours (Figure 6-5). Four classes were the least similar by their spontaneous activity and their response to a few compounds: the classes of ORNs in ab2, ab3 and ab4. Mainly, ab3A is less responsive to ripe fruit headspaces of orange and pb1A is more sensitive to attractive ripe fruit odours of blueberry and strawberry in *D. suzukii*. The ab1A was more activated by raspberry headspaces in *D. melanogaster*. The ab4A ORN class differed in the response to strawberry headspace and an increased affinity for grape in *D. melanogaster*, which was however not supported by a statistical comparison (appendix 9).

It can be concluded that ab3A and pb1A have undergo major functional changes which strongly influenced the detection of ripe fruit volatiles. The detection of non-host fruits (orange) was reduced, while the detection of host fruits was increased (strawberry and blueberry) in *D. suzukii*. The role of ab1A is unclear. It is a major ORN as it is highly activated in a similar manner in both species yet in *D. melanogaster* ab1A was more sensitive to raspberry odours, a favoured host by *D. suzukii* (Chapter 3).

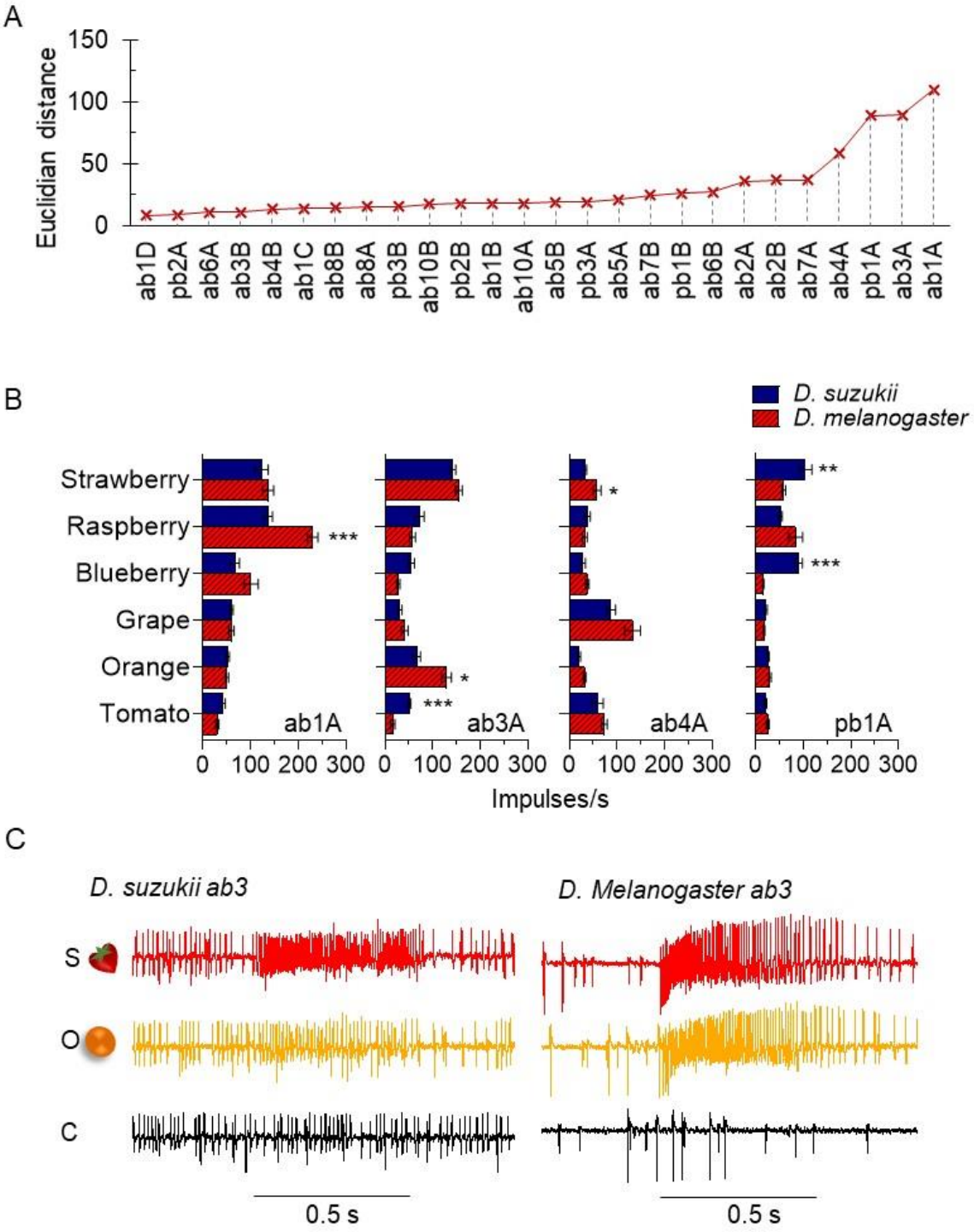


Figure 6-5 Ripe fruit detection in D. suzukii and D. melanogaster

A) Euclidian distance between homologous ORNs, calculated from the mean responses to six fruit headspaces. B) Mean (± SEM) response rate (impulses/s) to six ripe fruit headspaces by the four most dissimilar classes of ORNs (highest Euclidian distance) in *D. suzukii* (blue, plain) and *D. melanogaster* (red, striped). ***P<0.001, **P<0.01, *P<0.05, for significant differences (Appendix 9). C) Traces of the ab3 ORNs in response to a 0.5 s stimulus with strawberry (S, red), orange (O, orange) and ambient air (control, black) headspaces. The increased firing rate is from ab3A. Data for *D. suzukii* originated from chapter 3.

6.3.4 Affinity to fruit volatiles

Next, a similar discriminant analysis followed with statistical analyses permitted to compare both species on the tuning of ORNs to fruit volatiles, used for the characterisation of the functional basiconic types (Figure 6-6, appendix 9).

The classes of ORNs ab7A and B, ab3A ab10A, ab2B and pb1A were the most dissimilar between the two species. A statistical comparison of their responses to fruit volatiles highlighted few chemicals. Compounds that were significantly differently detected by more than one ORNs are: Isoamyl acetate, 2-heptanone and (*E*)- 2 hexenal. *D. suzukii* ORNs were of increased affinity for these compared to *D. melanogaster*. All ab3A responses were not significantly different between species despite larger responses in *D. suzukii* (with 100 impulses/s difference) to ethyl acetate and β-cyclocitral and a lower response to isoamyl acetate. Only the detection of 2,3-butanedione was significantly different.

Some large differences could not be statistically verified because too few recordings were performed on *D. melanogaster* (appendix 9), despite *D. suzukii* ORNs being more sensitive with more than 100 impulses/s. Notably, ab10A did not significantly differ in responses to volatiles, despite higher mean responses to pentyl acetate, isoamyl acetate, (*RS*)-1-octen-3-ol and ethyl lactate. The ab7A was also more responsive to (*RS*)-linalool.

It can be concluded that the classes of ORNs ab7A and B, ab3A ab10A, ab2B and pb1A have undergo functional changes leading to a differential affinity for fruit volatiles. Isoamyl acetate, 2 heptanone and (*E*)- 2-hexenal detection appear the most involved in host shift than any other. Pentyl acetate, (*RS*)-1-octen-3-ol and ethyl lactate may also be involved. The classes pb1A and ab3A appear as the most specialised ORNs for host selection in *D. suzukii.*

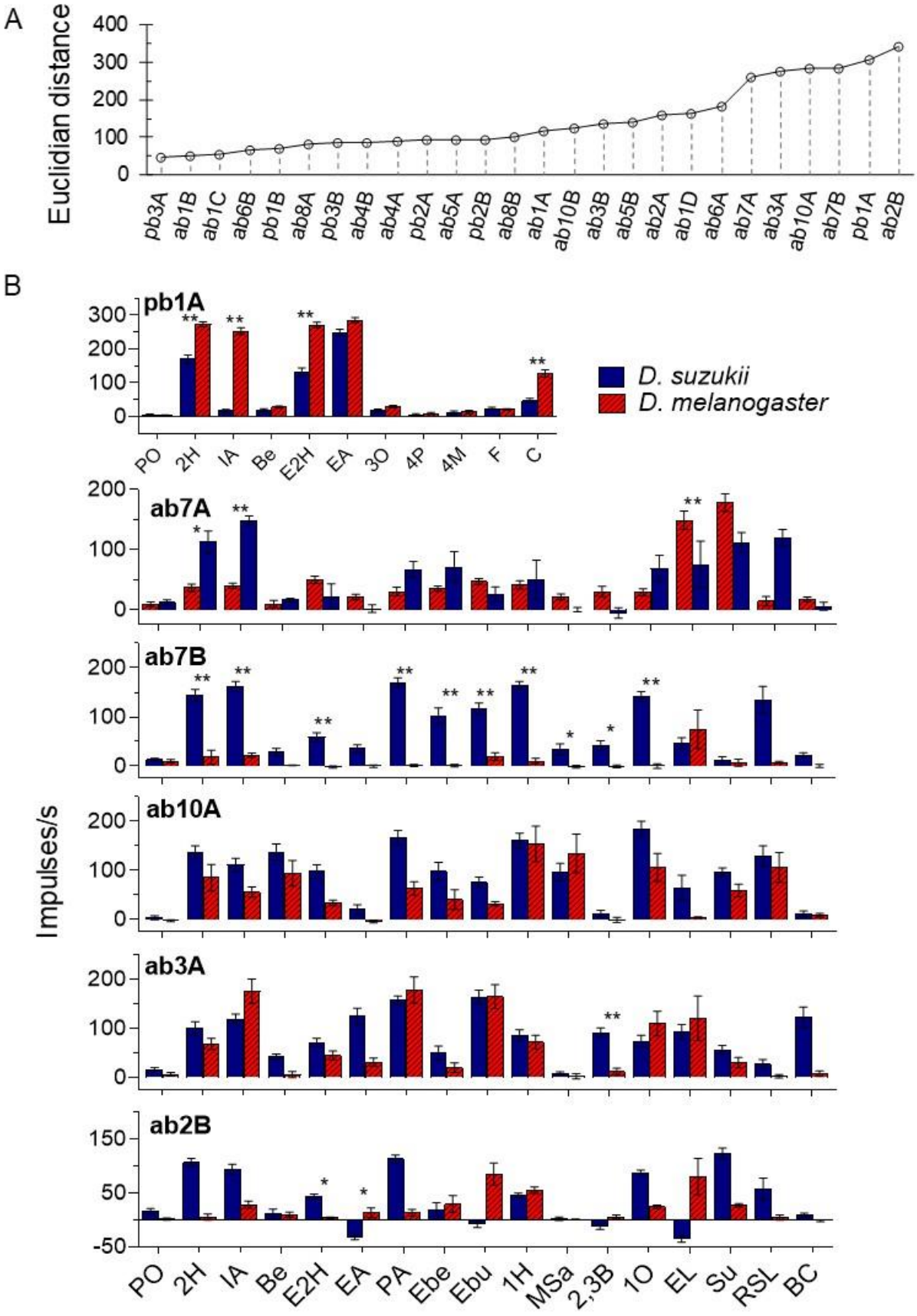


Figure 6-6 Functional differences of ORNs responses between D. suzukii and D. melanogaster

A) Euclidian distance between homologous ORNs, calculated from the mean responses to chemicals. B) Mean (± SEM) response rate (impulses/s) from the most dissimilar ORNs pb1A, ab2B, ab3A, ab10A, ab7A and ab7B for *D. suzukii* (blue, plain) and *D. melanogaster* (red, striped). Response rates recorded during a 0.5 s stimuli with 30 µl of a 1% solution in paraffin oil of 2-heptanone (2H), isoamyl acetate (IA), benzaldehyde (Be), (*E*)-2-hexenal (E2H), ethyl acetate (EA), pentyl acetate (PA), ethyl benzoate (Ebe), ethyl butanoate (Ebu), 1-hexanol (1H), methyl salicylate (Msa), 2,3-butanedione (2,3B), (*RS*)-1-octen-3-ol (1O),ethyl lactate (EL), 6-methyl-5-hepten-2-one (Su), (*RS*)-Linalool (RSL), β-cyclocitral (BC), 3-octanol (3O), 4-propylphenol (4P), 4-methylphenol (4M), fenchone (F), cyclohexanone (C). Control was with only paraffin oil (PO). Significant differences between species **P<0.01, *P<0.05 (Appendix 9). For all panels, data mined from chapter 3 and chapter 6.

6.3.5 Detection of repellent odours and role of ab10B

Using the model characterised in chapter 3 and chapter 4, it may be possible to identify repellent semiochemicals by testing their affinity for a repellent detecting olfactory circuitry. For this study nepetalactol was chosen as a candidate repellent odour because of its behavioural effect on *D. suzukii* (Ebrahim *et al.* 2015). The hypothesis tested is that different enantiomers of nepetalactol may induce different behavioural outcomes if the olfactory circuitries they activate diverge. The aim of the study was to determine how the pattern of activation of these enantiomers differ between species and possibly identify which are inducing avoidance behaviour from the parasitoid wasp. One of the nepetalactol enantiomer, known as a pheromone compounds of the Aphid genius: the (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (Dawson *et al.* 1987) was available for this study. Using a stimulus at the concentration of 1% on filter paper in paraffin oil the response to the (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol was collected for all antennal basiconic ORNs in both species.

The ORNs ab6A and ab3A were the most responsive to the compound, equally in both species with an increase > 150 impulses/s compared to the spontaneous activity. In *D. suzukii*, ab1A, ab3B, ab4A, ab7A and abXB were also activated with nearly 100 impulses/s. It appears that ab1A and/ or ab1D may have been activated but only one recording was not sufficient to be conclusive. The ab10B was only activated with about 70 impulses/s increase in *D. suzukii* and below 50 impulses/s in *D. melanogaster*.

It can be concluded that (1R,4aS,7S,7aR)-nepetalactol is detected by species specific subsets of ORNs and that *D. suzukii* appear overall more sensitive to it with seven classes of ORNs being strongly activated, against two for *D. melanogaster*. In both species, the detection was mostly mediated via ab6A and ab3A. In addition, the compound being associated with aversion, simultaneously activated ab7A and ab2B in *D. suzukii*. The ab10B involved in the detection of a racemic nepetalactol (Ebrahim et al., 2015), was not the most activated by this enantiomer in both species.

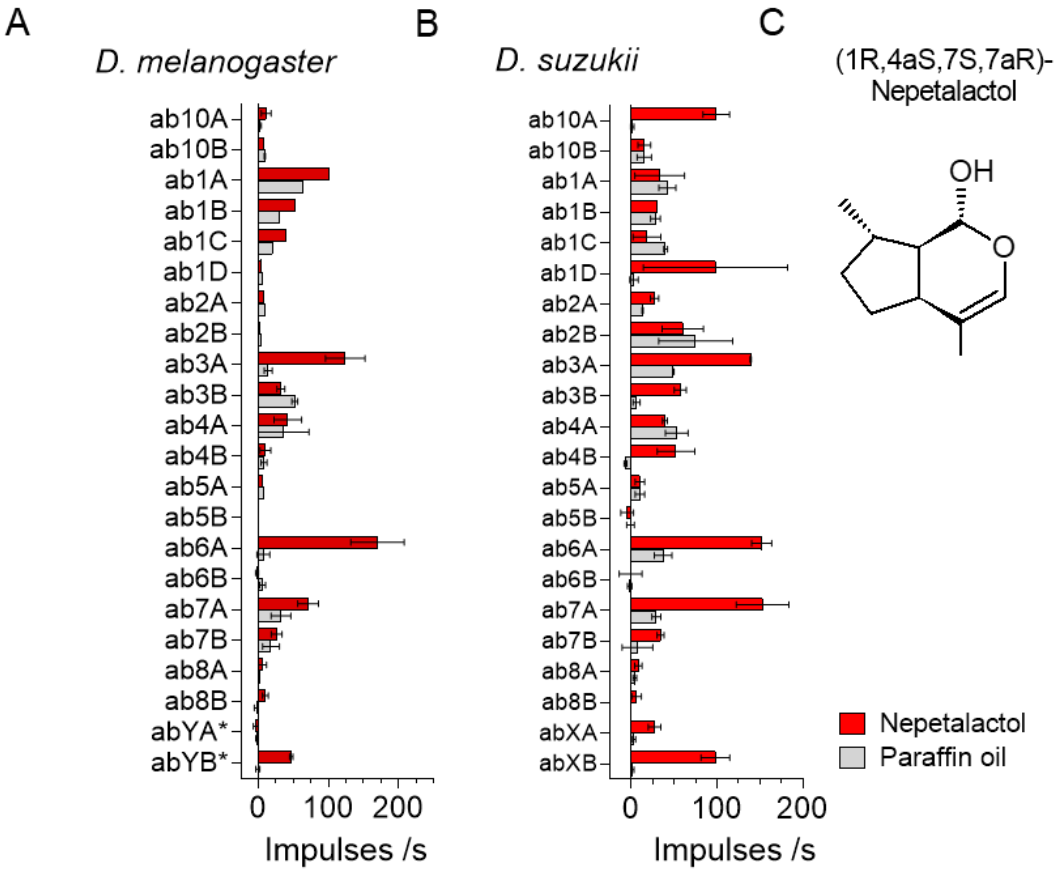


Figure 6-7. ORN responses to (1R,4aS,7S,7aR)-nepetalactol, in D. suzukii and D. melanogaster

Mean ± SEM response rate (impulses/s) during a 0.5 s stimulus with 30 µl of a 1% solution of (1R,4aS,7S,7aR)-nepetalactol (red bars) and the solvent paraffin oil (grey bars), in *D. melanogaster* (A) and in *D. suzukii* (B). N=2-10. C) 2-D conformation of the molecule.

6.4 DISCUSSION

Detection of the whole bouquet of ripening fruits via ORNs in *D. melanogaster* has not been studied much in comparison with fermenting and damaged (e.g. mashed, cut) fruit odours. Many fruit odours have been tested guided by different research questions, but no study looked at the whole ripe fruit detection on each ORN class. Using the same fruit selection as in *D. suzukii*, the fruitprints in *D. melanogaster* were collected in order to compare the two species. The aim of this comparison using fruit headspace was to determine whether the dissimilarities identified previously induced a species-specific detection of ripening fruit volatiles. Furthermore, a mechanism of olfactory discrimination of ripe fruit odours from taxonomically diverse plant species would be interesting to assess in this species which is also attracted to fruit odours in addition to yeasts. Is *D. melanogaster* able to discriminate among ripe fruit types? How is its perception of whole undamaged fruits that are not accessible for food and oviposition?

The same hypotheses on the encoding of fruit odours by the peripheral olfactory system (Introduction, 1.4) are validated in *D. melanogaster* and in *D. suzukii*. The larger differences between species are described and discussed in this study in light with the results from other chapters. Differences observed for the classes of ORNs which were most similar are also reported (appendix 9) and should be considered when additional evidence of the role of these ORN in fruit detection is shown.

6.4.1 Discrimination of fruit odours mediated by seven classes of ORNs

The present study demonstrated that only a subset of ORNs is activated by different fruits. These seven ORNs appear sufficient to enable the discrimination of six ripe fruits in *D. melanogaster*, as it was demonstrated in *D. suzukii* (Chapter 3). No behavioural responses were assessed it is therefore unclear which fruitprints are associated with attraction. Nonetheless, the classes of ORNs that are activated by these fruits were the same as in *D. suzukii*, with some differences in the intensity of activation.

As discussed in Chapter 3 for *D. suzukii* these results on *D. melanogaster* supports that not all ORNs are involved in fruit selection and the number of chemicals that are detected does not reflect how the peripheral olfactory system detects whole fruit headspaces, in contrast with the study from Dweck *et al.* (2018).

As further discussed in chapter 3, a background noise activity from other odours from the environment may induce activations of all ORNs (Cafaro 2016) and could mask any activation by fruit headspaces that would be below a certain threshold. The latter remains to be determined but was hypothesised to be of approximately 40 impulses/s following the

results of chapter 3 and 4, and above. It is therefore sensible to hypothesize for further research that a behaviourally relevant activation of ORNs is given by only a subset of seven ORNs, characterised by the studies in chapter 3 and the present.

6.4.2 Model of ripe fruit detection in *Drosophila species*.

6.4.2.1 Detection of citrus fruits

In this study, orange headspaces activated ab3A (with more than 50 impulses/s). As discussed in chapter 4 and introduced earlier, ab3A is associated with host attraction and host shift in *D. suzukii* (Keesey *et al.* 2015). In this study, the loss of activation of ab3A by orange headspaces in *D. suzukii* compared to *D. melanogaster* is a strong indicator of the role of ab3A in host shift as oranges were not attractive to *D. suzukii* (Chapter 3). It suggests that ab3A sensitivity for citrus fruits may have decreased in *D. suzukii* compared to *D. melanogaster*. However, it is unclear from this study whether females would be attracted to damaged citrus fruits, which flesh would be accessible for egg laying, and whether the fruitprint of damage fruits would be similar to one of whole fruit, and perhaps activating ab3A. Besides, it is unclear whether *D. melanogaster* would be attracted to whole undamaged ripe oranges.

Ab3A may not be the only one involved in a host shift. *D. melanogaster* was found attracted to citrus fruits via a dedicated pathway involving solely ai2A, detecting valencene (Dweck *et al.,* 2013). The authors did not report any activity from other ORNs, including ab3A. Ai2 was not found in this study, but the abXA in *D. suzukii* was the most responsive ORN class to ripe orange headspace. It could be hypothesised to be the homologous of ai2. However, only two co-localised ORNs were found in abX, against three reported for ai2 (Dweck et al 2013). Further characterisation is therefore required to identify ai2 and abX in both species.

6.4.2.2 Sensitivity to the fruit volatiles isoamyl acetate

Isoamyl acetate is associated with both ripe and overripe fruits (Stock *et al.* 2010), and can be attractive alone, as demonstrated in chapter 4 and by Revadi *et al* (2015). It was also found to be repellent when in mixture with additional decay associated odours (Cha *et al.* 2012; Piñero *et al.* 2019). *D. suzukii* sensitivity to isoamyl acetate is increased compared to *D. melanogaster*. This was shown from the recordings on all classes of ORNs using a 1% solution. Revadi *et* al. (2015) showed that the attraction to isoamyl acetate decreased with increasing doses. The present study bring evidence for a possible mechanism: higher doses of isoamyl acetate induced the additional activation of ORNs (notably ab7A). Their activation may decrease attraction to isoamyl acetate (Chapter 4). They also reported, along with Ramasamy *et al.* (2015) and Hickner *et al.* (2016) a positive selection for genes coding for ORs that are sensitive to isoamyl acetate. All together, these studies and the present

thesis confirm that isoamyl acetate must be a host detecting volatile and was detected in small amounts in strawberry headspaces (chapter 4). Yet, it was not shared by all hosts, only small amounts are attractive and its association with other chemicals may need careful consideration.

6.4.3 Functional changes associated with gene events

Ramasamy and colleagues (2015) identified the loss of Or85a expressed in ab2B (in *D. melanogaster*) as a replacement by another unidentified OR, in *D. suzukii*. Furthermore, Keesey *et al.* (2019) found a strong dissimilarity in ab2B responses to chemicals in *D. suzukii* compared to other *Drosophila* species. These dissimilarities are supported by a loss or change of function in the tuning of the ORs to fruit odours compared to *D. melanogaster* in this study. In addition, the increased number of ab2 sensillum type, hereby an increased number of ab2B in Chapter 3 suggest an important role of ab2 associated ORNs and ORs in host selection as discussed chapter 3 and 4. They are also tuned to a broader selection of ripe fruit odours compared to the ORNs in *D. melanogaster*. They may have increased in number and affinity in relation to the adaptation to a novel habitat. The ab2B activation by isoamyl acetate also suggested its role in attraction to fruits (chapter 4) even though it may have other roles, as associated with unattractive cues (chapter 4 and 5). ab2B appeared more inhibited by ethyl acetate in *D. suzukii* than in *D. melanogaster*. This may be due to the large spontaneous activity difference between the two species which enhance the inhibitory effect. It could also be a sign of crosstalk between the two co-localised ORNs, as discussed in Chapter 4. This hypothesis is also supported by the non-activation of ab2B by fruit headspaces as discussed chapter 3.

A loss of affinity for overripe fruit odours (i.e. ethyl 3-hydroxy-butanoate) and a gain in affinity for ripening fruit odours (e.g. 2-heptanone) were described (Keesey *et al.* 2015; Ramasamy *et al.* 2016) and supported by the present study with the increased affinity of ORs to fruit volatiles identified from ripening fruits: 2-heptanone, isoamyl acetate and β-cyclocitral, compared to *D. melanogaster*.

Lastly some functional changes measured by a comparative analysis of responses to fruit odours and to fruit volatiles are not correlated to genetic changes. Indeed, some genes that are under purifying selection, code for ORs expressed on ORNs that are functionally different in *D. suzukii* compared to *D. melanogaster*. For instance, the ab4A mainly responds to grape headspaces and to aldehydes in both species. ORN responses to the headspaces were different, yet their tuning to fruit volatile remained similar and the associated gene (*Or7a*) was conserved (Hickner *et al*. 2016; Ramassamy *et al*. 2016).

6.4.3.1 The role of ab3A

These three above compounds are associated with ripe fruit detection and were found relevant to *D. suzukii* by Keesey *et al.* (2015) and Revadi *et al* (2015). The ORNs ab3A strongly differed between species via their response to β-cyclocitral. Here, they were not significantly different ($P > 0.1$) despite large sample sizes. The reason for this is the presence of flies in the *D. suzukii* population whose ab3A responded as little as the ab3A in *D. melanogaster,*to this compound (Chapter 7). The recordings of many ab3s led to the identification of two subtypes of ab3 seemingly found on distinct flies. A deeper analysis of this discovered phenomenon is addressed In Chapter 7 along with detailed description of the responses to all stimuli used in this work.

6.4.3.2 The role of pb1A

The present study highlighted a higher sensitivity to ripe fruit odours from pb1A in *D. suzukii* compared to *D. melanogaster*. Furthermore, in chapter 4, its activation in addition to ab1A induced attraction of gravid females *D. suzukii*, with a small increase of activation (about 50 impulses/s). As discussed in chapter 4, this pb1A activation appear to mediate attraction and a crucial part of the olfactory circuitry which enable females to select and discriminated host substrates. The pb1A and other maxillary palp ORNs have been associated with the detection of many compounds from fruit odours in *D. melanogaster* (Dweck *et al.* 2016). The techniques used for headspace collection and for electrophysiological recordings differed, as the author did not record the response to the whole extract but to the compounds from the extract. Furthermore, the authors reported similar electrophysiological responses from these ORNs in both species in response to compounds from fruit extracts by GC-SSR. The fruits and compounds identified differed from this study. In light with the present study, it appears that responses to fruit volatiles does not necessarily reflect the response to the headspace: only pb1A from the maxillary palp in *D. melanogaster* responded to fruit headspaces in this study, yet all pb1-pb3 ORNs responded to some of the individual components in the study by Dweck *et al*. (2016).

6.4.4 The possible roles of ab6A ab7A, ab10B in detecting repellent odours

The identification of sensory units involved in the detection of natural enemies would allow to identify candidate masking and repellent odours by a screening of their induced activity on these sensory units. These ligands may be used as signal disruption in the fields. Indeed, the ability of the flies to detect enemies would be impaired, the flies would thus be unable to detect the parasitoid wasps that are purposely released in great numbers for biological control. This could help improve management of *D. suzukii* as part of IPM.

The present study demonstrates that a single enantiomer of nepetalactol, (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol activated a species-specific combination of ORNs. In both species the ab6A and ab3A were the most sensitive classes. *D. suzukii* was the most responsive with five classes of ORNs being activated with more than 100 impulses/s increase from the spontaneous activity. In *D. melanogaster* only the classes ab6A and ab3A were as much activated.

The racemic mixture of nepetalactol, was detected solely by ab10B, and specifically mediated by Or85f when Ebrahim and colleagues (2015) characterised the ORN responses to *Leptopilina heterotoma* body odour compounds, in both *Drosophila* species. Reversely, in the present study ab10 ORNs were not activated by nepetalactol in either species. Having found a very different result using one enantiomer in this study, two hypotheses may explain the difference.

6.4.4.1 The characterisation of ab10B

First, it appears that the ab10 identified in chapter 3, did not match the description of the ab10 identified and tested by Ebrahim *et al.* (2015). Different panels of ligands were used. The authors identified the functional type ab10 via a strong response of one of the ORNs to methyl benzoate and a strong activation of ab10A by phenethyl alcohol in *D. melanogaster* but not in *D. suzukii*. The class ab10A selected in the present study was simultaneously activated by 2-phenylethanol and diethyl succinate in both species (Appendix 9). The ab10 as identified in this study appeared functionally conserved between species, with only a small decrease in response to grape headspaces in *D. suzukii*. It can be suggested that a difference in the results between the study of Ebrahim and colleagues (2015) and the present is because of different classes of ORNs being recorded from. Yet, the ab10 identified from *D. melanogaster* appear to be the same and in the present study, ab10B was not activated by nepetalactol in *D. melanogaster*.

6.4.4.2 The racemic mixture is not the sum of the enantiomers

The second hypothesis is that a racemic mixture is detected differently from each enantiomer. The enantiomer was identified as part of a racemic mixture of nepetalactol from body wasp odours of *L. heterotoma*. by Ebrahim *et al.* (2015). It is possible that the mixture of enantiomers have a different pattern of activation than the compounds alone (Introduction, 1.2.2). Enantio-specificity was found in the detection of linalool and discussed in chapter 4, hereby supporting this hypothesis.

Unlike the study from Ebrahim *et al*. (2015), not only one ORN class was activated, but many. Additional studies, notably behavioural assays would permit to highlight the roles of these species-specific combinations of ORNs and particularly of the ab6A which was the most activated by this enantiomer in both species.

6.4.4.3 The ab7A ORNs: conserved mechanism for aversive behaviour

ab7A was associated with non-host substrates and unattractive odours in chapers 3, 4 and 5 of this thesis, in *D. suzukii*. Chapter 4 permitted to highlight that activating ab7A reduced the attractiveness of fruits. In chapter 4 and 5, ab7A activation and the number of its ligands was greater in the headspaces of unattractive (and unsuitable) fruits and cultivars than in the attractive and suitable ones.

The change in sensitivity of ab7A associated ORN is also demonstrated by a larger dissimilarity between *D. melanogaster* and *D. suzukii*. It can be hypothesised that ab7A may play a role in the shift to ripening fruits, by decreasing the attractiveness of non-host fruits, such as fruits which skin cannot be pierced (grape cultivars, tomato) and overripe and damaged fruits (detecting volatiles associated with these stages).

The activities of the two co-localised ab7 ORNs were difficult to tell apart because of similar amplitudes of their action potentials (not shown) on electrographs. The quantification of responses was thus challenging. The responses of ab7A and ab7B may have been mixed and additional experiments, perhaps with single OR expression (Dobritsa *et al.* 2003) would enable to differentiate the two responses. It is clear nonetheless that ORNs in the ab7 functional basiconic type are of increased sensitivity to fruit odours in *D. suzukii* compared to *D. melanogaster* and are associated with non-attraction and masking of attractive odours.

Lastly, the ab2B appears to have underwent similar changes. Its response to fruit volatiles diverged most from *D. melanogaster* and, as discussed chapter 3, 4 and 5, it may have a significant role in host selection which remains to be deciphered. These results are supported by the results of Keesey *et al.* (2019), which also reported a functional change for ab2B.

6.4.5 The abX and abY types

The two types abX in *D. suzukii* and abY in *D. melanogaster* were not described before and did not share enough common ligands. The abX was also described recently by a response to linalool by Keesey *et al.* (2019). The authors considered it to be the ab9. Given the non-similarity of abX in this study and the description in literature of ab9 (as reviewed above), the types were kept distinct until additional characterisation. The ab9 was not found in *D. melanogaster* in this study and could therefore not be compared. This functional type has been associated with two ORNs carrying the ORs Or67b, Or69aA and Or69aB (isoforms of Or69a) by Couto *et al.* (2005). The description of their function was done by extracellular recordings on sensilla containing neurons expressing a single target OR in *D. melanogaster* (Dobritsa *et al.* 2003). These were found to mediate species-specific attraction to both sex and food odours (Table 1-1). It was further suggested that these isoforms may play an

important role in speciation when the dual affinity was not found in other drosophilids (Lebreton *et al*. 2017). Homologous genes to the genes coding for ab9 associated ORs were found by Hickner *et al.* (2016) and Ramasamy *et al.* (2015) in *D. suzukii* and did not appear to have undergo any evolutionary changes (Table 1-1). Furthermore, a BLAST of sequences between the NCBI and the *Drosophila suzukii* genome database, Spotted Wing Fly Base enabled to identify homologous genes coding for the ORs located in ab9 types (not shown), supporting the sequencing and phylogenetic work. The ab9 ORNs are therefore likely to be present on the antenna in *D. suzukii* and may have an important role which the present study could not approach.

The abX in *D. suzukii* represent an interesting new type to investigate. Linalool is a terpene found in multiple fruits and associated with attractiveness, notably in *D. melanogaster* (Dweck et al., 2013) but its attraction in *D. suzukii* remains unclear (Chapter 4). Further characterisation is required to identify which ORs may be associated with the ORNs housed in abX and abY. They may be compared to the uncharacterised types in *D. melanogaster* ab11, ab12 by Kwon *et al.* 2011, and perhaps ai1-ab3 by Dweck *et al.* (2013), the latter being associated with detection to citrus fruits.

6.5 *CONCLUSION AND FUTURE DIRECTIONS*

The results of this chapter demonstrate that the peripheral olfactory system in *D. melanogaster* is similarly tuned to ripe fruit odours as in *D. suzukii*: a subset of seven classes enable the discrimination of six ripe fruits from taxonomically diverse plant species. This model supports the hypotheses which were addressed in this thesis. The classes of ORNs which were previously shown as determinant for host selection in *D. suzukii*, functionally diverged in their response to ripe fruits from *D. melanogaster*. Combinations of these ORNs enable gravid females of both species to discriminate fruits by the plant volatiles bouquets. Not all ORNs responding to individual fruit volatiles are necessary for the encoding of fruit headspaces.

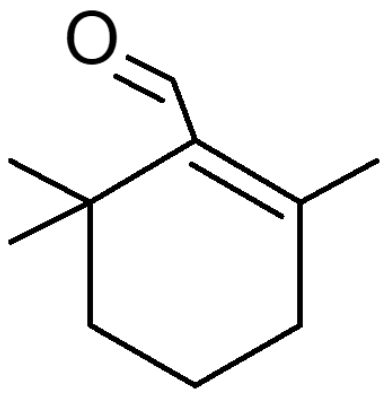
The role of ab10B in the detection of nepetalactol is contradictory to earlier findings. Additional clarifications are needed in order to characterise the response patterns to repellent cues that appear to be mediated by more than one olfactory circuitry in this study, involving ab6A.

Lastly, the previously characterised role of ab3A in host specialisation in Drosophilids was diminished by all results presented in this thesis. Changes in the detection of ripening fruit selection in *D. suzukii* were clear from this chapter and the ab3A was activated by the most attractive fruit headspaces and by a majority of fruit volatiles (Chapter 3 and 4) yet, its activation did not appear necessary to induce attraction. Its role as a single olfactory circuitry or as a combination requires to be further investigated. An additional finding from the work carried out in this research was that not all *D. suzukii* adults responded similarly to the leaf volatile β-cyclocitral by ab3A, an olfactory pathway highlighted by Keesey *et al*. (2015).

The following question is addressed in the next chapter (Chapter 7) of this thesis: how important is the detection of β-cyclocitral by ab3A for host selection?

CHAPTER 7

7 IMPAIRED DETECTION OF β-CYCLOCITRAL IN *DROSOPHILA SUZUKII*



2D conformation of β-cyclocitral

7 IMPAIRED DETECTION OF BETA-CYCLOCITRAL IN *DROSOPHILA SUZUKII*

7.1 INTRODUCTION

7.1.1 Aim

β-cyclocitral is a volatile associated with ripening fruits within the foliage of trees. Detection and attractiveness to this volatile have been suggested to have a role in the evolutionary shift from damaged and/or overripe (fallen) to ripening (within the plant foliage) fruits in *D. suzukii*. The data collection and analysis in Chapter 3 revealed intraspecific variation in how the olfactory receptor neuron (ORN) ab3A respond to this chemical in *D. suzukii*. Antenna of about 40% of the females in a laboratory population had ab3A which did not respond to β-cyclocitral with the same intensity as the other 60%. Two flies out of more than 50 for which the responses were recorded from, had both types of ab3A.

This chapter present the results of a study which aim was to characterise and attempt to understand the reasons behind the existence of two types of adult *D. suzukii* in the laboratory population. The hypothesis addressed was that the impairment of the detection of β-cyclocitral in *D. suzukii* is associated with the functional divergence of ab3A thereby affecting the detection of host fruits.

The occurrence of the so-called ab3-high and ab3-low ORNs in laboratory and field collected populations was assessed. Data mining from chapter 3 permitted to describe the ORN responses to fruit odours by ab3-high and ab3-low flies. A tentative selection of individuals on the criteria “response to β-cyclocitral” was made for further molecular analysis.

7.1.2 Background

7.1.2.1 The olfactory system in D. suzukii

In *D. suzukii* the peripheral olfactory system house 28 classes of olfactory receptor neurons (ORNs) dedicated to the detection of food/ host odours (Introduction,1.3). Pairs or quadruplicates of these ORNs are localised in basiconic sensillum structures forming functional types on the surface of the third antennal segment and maxillary palps of the fly. ORNs are associated with olfactory receptors (ORs) that can be tuned to few or many food/host volatiles. These ORNs once activated, relay sensory signals towards centralised olfactory units. Single sensillum recordings (SSR) enable to record extracellularly the activity of the ORNs in response to an olfactory stimulus through the insertion of an electrode in the sensillum lymph. This technique permitted to characterise the classes of

ORNs in *D. suzukii* and study their responses to host fruit odours (chapter 3). ORNs are labelled after the functional basiconic type holding them and the relative amplitude of their action potentials, compared to their co-localised neighbours on electrographs. For instance, the ab1 sensillum type house the classes of ORNs: ab1A, ab1B, ab1C and ab1D (Method, 2.8.2).

7.1.2.2 The ab3A and its response to β-cyclocitral

The first description of the response to β-cyclocitral, by the ab3A ORNs in *D. suzukii* was done by Keesey *et al.* (2015). It appeared to be involved in the attraction to ripening fruits by *D. suzukii*, and was suggested to be associated with host specialisation, as it is not detected by *D. simulans* nor by *D. melanogaster* which are not found around ripening fruits. They identified the compound from strawberry leaf headspaces. The compound was identified from headspaces of ripe whole orange fruit headspaces but not from strawberry fruit and plant in this thesis (Chapter 4).

The ORs Or22a and Or22b, which are associated with ab3A, have been described in *D. suzukii*. They are homologous of the ones in *D. melanogaster* (Hickner *et al.* 2016, Ramasamy *et al.* 2015). The detection of β-cyclocitral was believed to be mediated by the Or22a probably because of the narrowed range of ligands found for Or22b (Keesey *et al.* 2015).

An evolutionary pressure may be occurring on Or22a and Or22b. Indeed, Or22a along with ab3A are believed to be associated with host shift and host specialisation in *D. suzukii* (Keesey *et al.* 2015) and other Drosophilids, including *D. melanogaster* (Mansourian *et al.* 2018) , *D. orena* (Comeault *et al.* 2017), *D. sechellia* (Dekker *et al.* 2006) and *D. erecta* (Stensmyr *et al.* 2003). The ab3A ORN class was associated with attraction to ripe fruits in *D. suzukii* (Chapter 3) but did not appear to be necessary and sufficient to induce attraction (Chapter 4). A comparison to *D. melanogaster* highlighted only a few differences in how ripe fruit odours and volatiles were detected by ab3A, most of which were not statistically significant (Chapter 6). These differences appeared correlated to a divergent attraction to citrus fruits, but a role in a divergence from overripe to ripe fruit remained unclear.

7.2 *IDENTIFICATION OF TWO SUBPOPULATIONS OF D. SUZUKII BY THEIR RESPONSE TO BETA-CYCLOCITRAL*

Using ten females and nine males (one died), the responses of 3-6 ab3 ORNs per individual to β-cyclocitral, paraffin oil (solvent, negative control) and ethyl butanoate (strong ligand, positive control) were recorded, using a chemical standard stimulus (30 µl of 10^{-2} µl /ml or 1% v/v solution in paraffin oil). This led to the discrimination of two types of flies separated by their response to β-cyclocitral.

7.2.1 The ab3-high and ab3-low adult *D. suzukii*

A major difference in firing rate of the ab3A neuron during and following a 0.5 s stimulus with β-cyclocitral was characterised between individuals and compared to the ligand ethyl butanoate. Ethyl butanoate elicited a high response from ab3A ORNs that was consistently found in all flies. The ab3B neuron responded overall higher to β-cyclocitral than ethyl butanoate, but with no characteristic difference as seen for ab3A (Figure 7-1).

The difference between the firing rates in response to the three stimuli permitted to discriminate flies which ab3 responded with “high” intensity and flies responding with “low” intensity. The responses fell into two categories: lower and higher than half the maximal response of the ab3A neuron. Hence, the threshold of “half the maximal response” was adopted (Figure 7-1). Flies which ab3A response to β-cyclocitral was below or equal to this threshold were categorised as “low” responders (ab3-low) and the others as “high” responders (ab3-high).

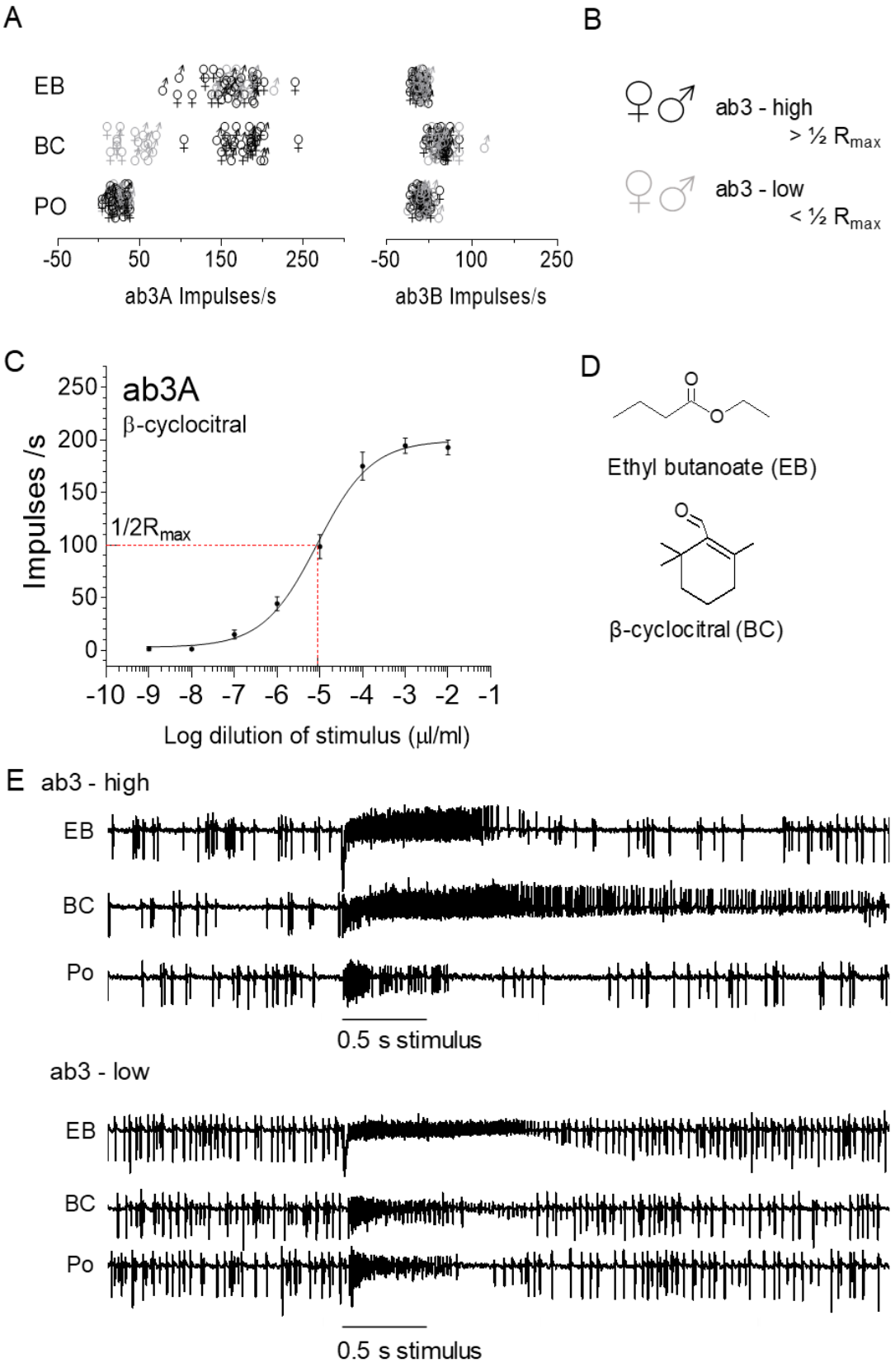


Figure 7-1 Characterisation of ab3A responses to β-cyclocitral in D. suzukii

A) Number of impulses/s during a 0.5 s stimulus with ethyl butanoate (EB), β-cyclocitral (BC) and paraffin oil (PO) by 22 females (female sign) and 11 males (male sign) ab3A

(left) and ab3B (right). In light grey, the responses categorised as ab3-Low and in black, the responses categorised as ab3-High. B) Flies were categorised by the responses of ab3A to β-cyclocitral: R> $1/2R_{max}$, ab3-high; R<$1/2R_{max}$, ab3-low. C) Dose-response relationship of ab3A and β-cyclocitral collected by W. van der Goes van Naters. A 4-parameter sigmoid Hill curve was fitted: $Y=R_{min} + (R_{max}-R_{min})/ (1+(10^{(LogD_{EC50}-X)}) *n_H)$ with R the response rate (impulses/s), D_{EC50} the dose which gives half the maximal response (R_{max}), and n_H the Hill coefficient (or slope). Half maximal response ($1/2\ R_{max}$) showed with dotted red line. D) ethyl butanoate and β-cyclocitral 2D conformations) E) Traces of ab3-high (upper) and ab3-low (below) to ethyl butanoate (EB, positive control), paraffin oil (PO, negative control and solvent), and β-cyclocitral (BC) during a 0.5 s stimulus.

7.2.2 No sexual dimorphism

The length of response of ab3A and the response by the ab3B were similar among individuals. All flies tested in this thesis (more than 50) displayed either the ab3-low or the ab3-high phenotypes, except two males found with both ab3 types on the same antenna. Both categories were equally found in males and females with approximately 60% ab3-high. The intensity of responses in ab3-high were similar for both sexes. The ab3A response to β-cyclocitral is approximately 30 impulses/s higher in ab3-low males compared to ab3-low females while the opposite is observed for the ab3B response (Figure 7-2).

7.2.3 Proportions found in population

To determine whether these two types naturally occur or originate from a laboratory selection three field populations were collected and studied along with the laboratory population in which the subgroups were originally found (Figure 7-2). Responses to β-cyclocitral and ethyl butanoate were recorded for ORNs in three or more ab3 sensilla from 20 flies originating from populations collected in Italy, United Kingdom, The Netherlands and the laboratory strain.

The 20 flies sampled from UK and Italy, all classified as “ab3-high” (having ab3-high only). In the population originating from The Netherlands, approximately 40% of the flies tested were “low” and 60% were “high”, the same ratios found in the laboratory rearing. It can be concluded that field populations are constituted by ab3-high flies. Further study will permit to determine whether the NL population was mixed with the laboratory population, or whether ab3-Low can occur in wild populations.

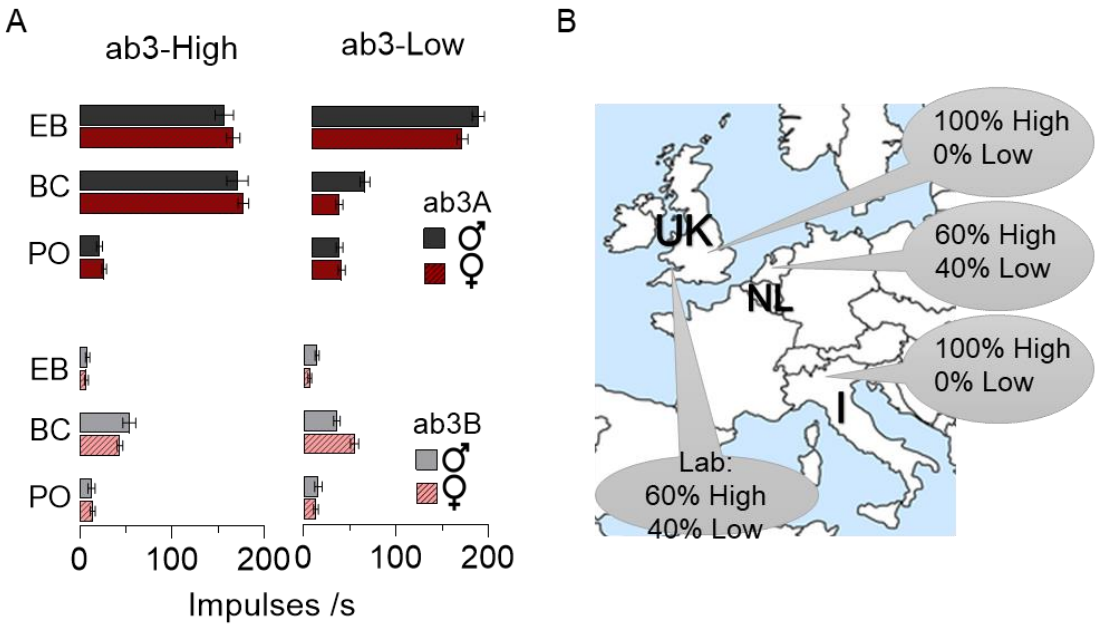


Figure 7-2 Distribution of ab3-high and ab3-low in the population

A) Mean (± SEM) response rate (impulses/s) of ab3A and ab3B from ab3-high and ab3-low male and females during a 0.5 s stimulus with 30 µl of a 1% solution of ethyl butanoate (EB), β-cyclocitral (BC) and paraffin oil (PO). B) proportions of ab3-high and ab3-low found in three field collected population in Italy (I), England (UK), The Netherlands (NL) and the laboratory population (Lab).

7.3 *Characterisation of responses from ab3-low and ab3-high in female D. suzukii: role in fruit detection*

The aim of this next study was to determine whether the impaired detection of β-cyclocitral impacted the detection of ripe fruits. Therefore, the responses to fruit headspaces and fruit volatiles were compared between the two types of flies. Data from chapter 3 and 4 were used and responses from ab3- low and ab3- high females were isolated. Not all females used in these chapters were characterised for their ab3 responses to β-cyclocitral, hence the sample size was reduced. The dissimilarities in responses were compared by geometrical distance (Euclidian distance) and statistical analysis of variance (method, 2.8.2).

7.3.1 The responses to ripe fruit odours were not impaired

The Euclidian distance between classes of ORNs was calculated from their responses to six fruit headspaces (Chapter 3). The responses of ab3 ORNs to fruit headspaces was similar for both subtypes (Figure 7-3) and these similarities were statistically supported (Appendix 10).

It can be concluded that ab3-high and ab3-low flies detected ripe fruit odours via a similar pattern of activation of ORNs on the peripheral olfactory system. This indicate that the non-detection of β-cyclocitral by ab3A (and all associated changes) did not impair host selection.

The Euclidian distance between homologous ORN in *D. suzukii* and *D. melanogaster* in responses to fruit headspaces was determined in chapter 6 revealing interspecific differences which were particularly large for the response of ab3A. A comparison with the Euclidian distance between lines revealed that the differences observed between lines were small compared to the difference between species (Figure 7-3). It can be concluded that the impairment of ab3A to detecting β-cyclocitral does not appear correlated with interspecific differences, thus with a host shift in the Suzukii lineage.

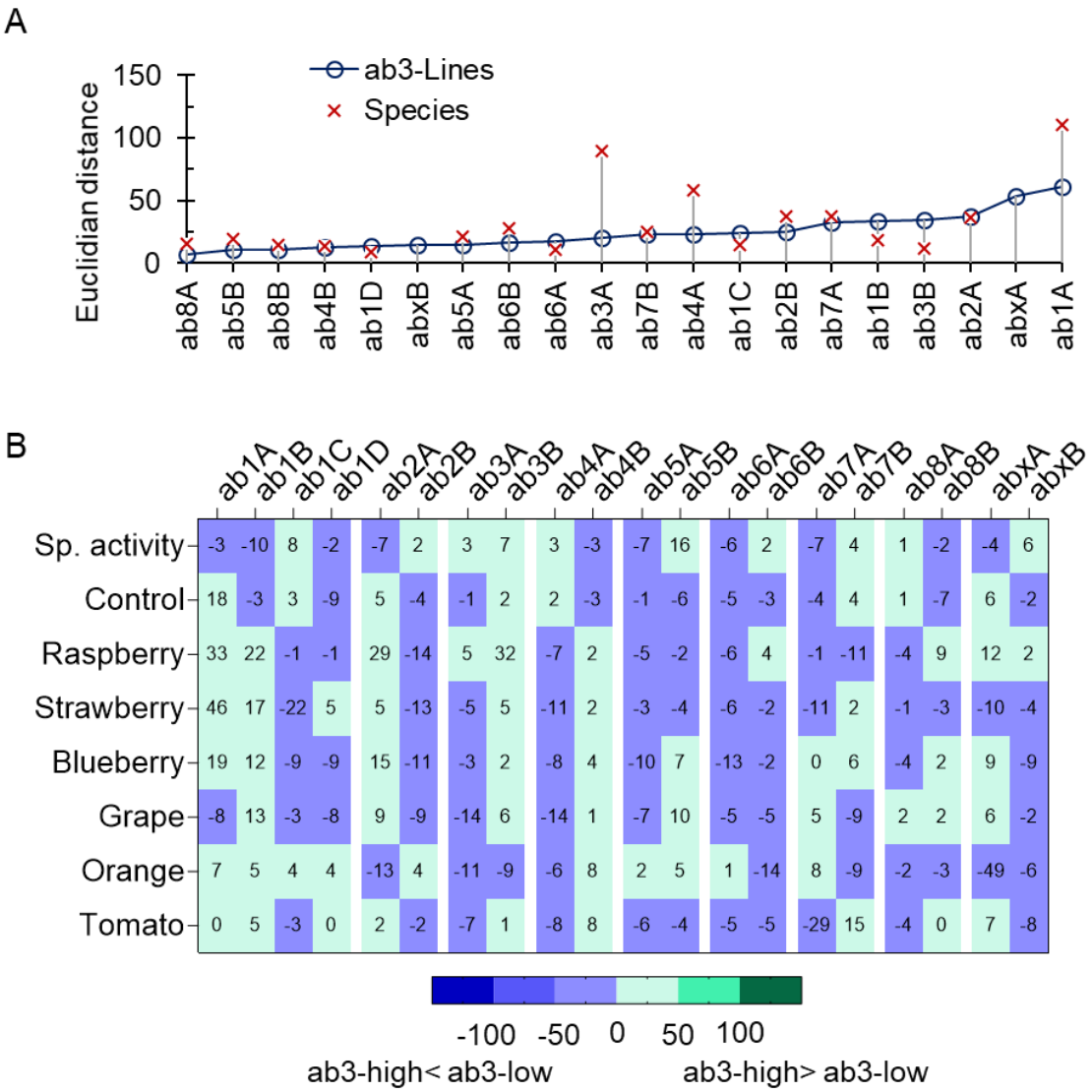


Figure 7-3 Similarities in response to fruit headspaces in ab3-high and ab3-low females D. suzukii

A) Euclidian distance between homologous ORNs of ab3-high and ab3-low lines of *D. suzukii* (Blue circles) and of *D. suzukii* and *D. melanogaster* (red crosses), calculated from the mean responses to six fruit headspaces B) Difference between mean response rates (impulses/s) of ORNs to fruit volatiles between high and low lines calculated as follow: $D_{h\text{-}l} = R_{high} - R_{low}$. With R the mean response rate from ab3-high and ab3-low during a 0.5 s stimulus with 0.01% dose of chemical. Data originated from chapter 3 and 6.

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7.3.2 Similarities in the detection of fruit volatiles

Next, the aim was to determine whether the changes in ab3A detection of β-cyclocitral is associated with other changes as for instance the detection of other chemicals. This may enable to identify whether the mechanisms at the origin of the ab3A impairment is specific to ab3A or affect other olfactory circuitries. The responses of all ORNs to chemicals characterised from fruits (chapter 4) were separated by lines in order to compare the ab3-High and ab3-low flies. Comparisons were only possible for ab1, ab3 and ab4 ORNs with small sample sizes, however several chemical classes were represented: esters, alcohol, terpenes, acids and aldehydes (Figure 7-4).

The response to the fruit compounds which were tested on both lines using a dilution of 0.01% (as in Chapter 4) did not differ between *D. suzukii* lines. This result was supported by a Wilcoxon summed ranked test but, due to the small sample size some differences may have been underestimated by the statistical analysis. It is particularly true for the response of ab3A to β-cyclocitral with approximately 72 impulses/s more for ab3-high than ab3-low, the difference which distinguish both lines. Responses from ab3A to pentyl acetate, prenyl acetate and methyl hexanoate were the next most divergent with a decrease in the response rate of 50 impulses/s for ab3-high. From this panel of volatiles at a dose of 0.01%, it can be concluded that only few ab3A responses diverged between ab3-high and ab3-low lines, none of which were as high as the different responses to β-cyclocitral. Other ORN responses did not appear different.

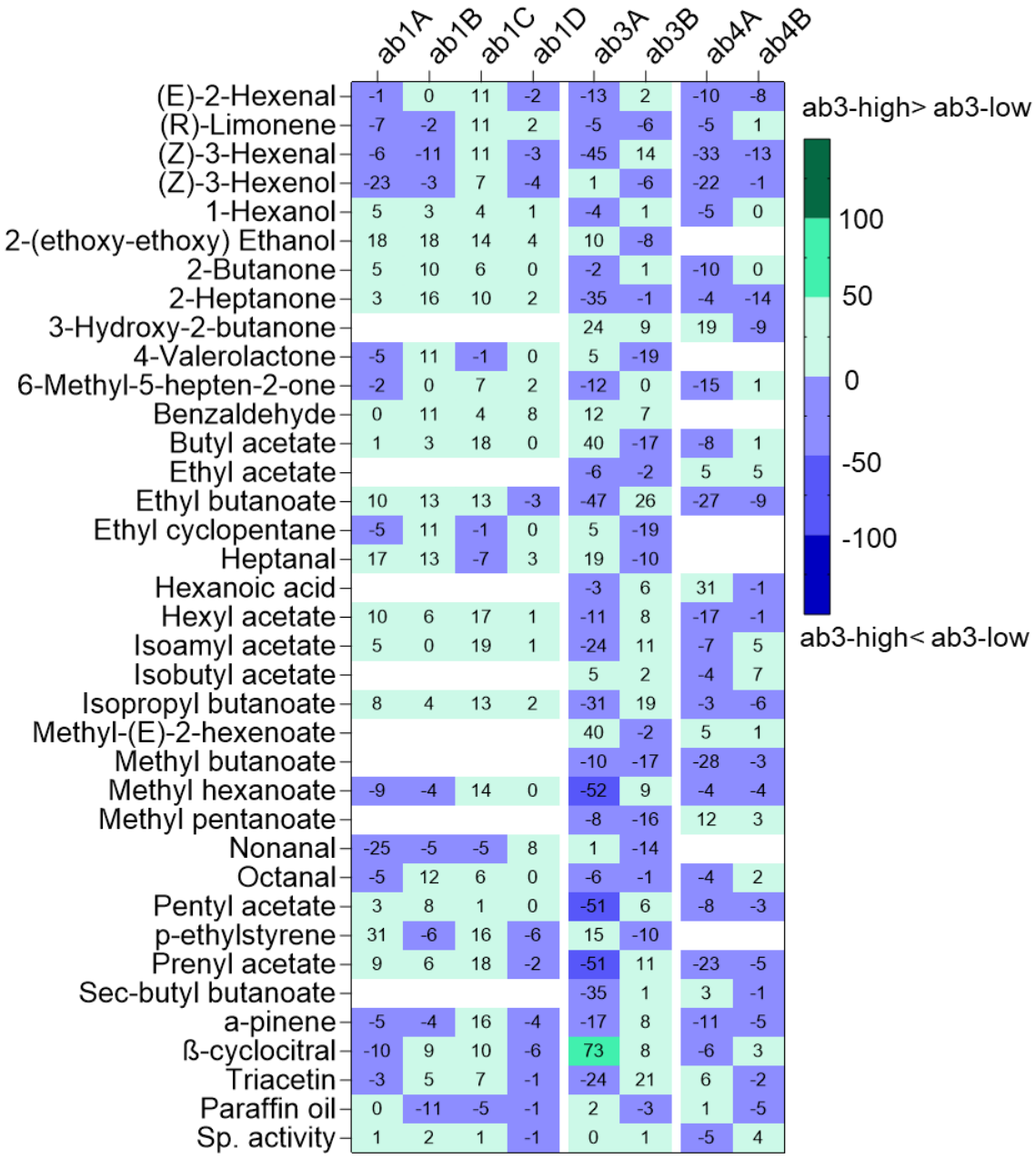

Figure 7-4 Fruit volatile detection difference between ORNs of ab3-high and ab3-low female D. suzukii

Difference between mean response rates (impulses/s) of ORNs to fruit volatiles between high and low lines calculated as follow: $D_{h\text{-}l} = R_{high} - R_{low}$. With R the mean response rate from ab3-high and ab3-low during a 0.5 s stimulus with 30 µl of a 0.01% solution in paraffin oil. Data originated from chapter 4.

7.4 DISCUSSION

While proceeding with the characterisation of the classes of ORNs on the antenna (Chapter 3) and after comparison with *D. melanogaster* (Chapter 6) a noticeable intraspecific difference in response of ab3A to β-cyclocitral was noted: two sub-population, so called ab3-high and ab3-low were characterised because all ab3A responded wither highly or weakly to β-cyclocitral. In the present study, only two flies out of more than 20 recorded from were found with both types of ab3 on a same individual. These ab3-low were not found in two out of three field collected population. Its presence in the collection from the Netherlands will need to be further investigated. It therefore appeared that these two lines separated within a laboratory rearing.

A latter publication revealed the independent identification of these two types of ab3. They were observed on separate and on a same animal in a laboratory population from the *Drosophila* Stock Center (USA) by Keesey and colleagues (2019).

The present study aim was to characterise the two types of responses and identify whether other olfactory responses differed between the two subtypes. The work used data collected in chapters 3 and 4 and implied that animals were either ab3-high or ab3-low, as it was most of the cases encountered. The occurrence of both types will need further investigation.

7.4.1 The ab3A neurons and β-cyclocitral are not necessary for host selection

This leaf volatile was associated with host selection in *D. suzukii* (Keesey *et al.* 2015), The impairment in its detection did not influence the detection of ripe fruit headspace volatiles, hence host selection. The ab3A that did not respond as much to β-cyclocitral (i.e. ab3-low) seemed overall more sensitive to other ligands, particularly pentyl acetate, prenyl acetate and methyl hexanoate. However, only a selection of odours was used with variable sample sizes hence, other affected ligand specific detection may have been missed.

The study also looked at ab1 and ab3 responses to fruit volatiles in both subtypes with a small sample sizes, hence results must be considered as informative. It appears that the activation of ab3A by isobutyl acetate was high and not significantly different, which contradicts the result from Keesey *et al*. (2019) showing this compound to be detected only by the so-called ab3 type ii (ab3A-low). However, the dose of chemical used for the stimuli were higher in their study. A difference in sensitivity at higher doses is therefore likely.

The ab3A was also associated with host specialisation in four *Drosophila sp.* including *D. suzukii* in several studies. Notably, through the detection of β-cyclocitral by Keesey *et al.* (2015). Indeed, ab3A was identified as inducing attractiveness of flies and associated with

a host specialisation in *D. melanogaster* (Mansourian *et al.* 2018), *D. orena* (Comeault *et al.* 2017), *D. sechellia* (Dekker *et al.* 2006) and *D. erecta* (Stensmyr *et al.* 2003). This leaf volatile, enhanced attraction of flies in field trials (Keesey *et al.* 2015, Pineto *et al*. 2019). Having not tested its behavioural effect, it is not possible to conclude whether gravid females do use this volatile as a cue to find oviposition substrates. However, the present study suggests that to detect the oviposition substrate, this volatile is not necessary as the fruitprints enabling the flies to discriminate suitable oviposition sites were not changed in flies which did not detect β-cyclocitral. This volatile was identified only from orange headspace (Chapter 4) and having not used leaf material to create fruitprint, the detection of β-cyclocitral may however been overlooked in this thesis.

Two attempts at creating two lines of *D. suzukii* as for ab3-high and ab3-low were unsuccessful in this study. Couples (1 male and 1 virgin female) were kept in individual vials for at least seven days before each fly was tested with SSR. Each generation, couples of two responding or two non-responding flies were made until each population was composed exclusively of either responding or non-responding flies. It is possible that more flies carried both ab3 types and were overlooked, hence the separation of the two types was not possible. Because the two ab3 types have been described on both flies separately and on a same animal, the origin of the impairment might come from different mechanisms. For instance, a mutation on associated *OR genes, a splicing* or an epigenetic regulation of OR expression have been shown to occur in *Drosophila* (Ray *et al.* 2007; Sim *et al.* 2012). It is also unclear whether these two types follow the same circuitry (i.e. innervates the same glomerulus) (Keesey *et al.* 2019).

7.4.2 Conclusion and future directions

A first step in deciphering the origin of the impairment, was to compare the Or22a, Or22b genes and their regions, between ab3-high and ab3-low flies. The sequences could be compared between lines and with annotated published sequences. This region of approximately 6000bp was amplified using a set of primers from flies that had been selected from the fourth generation as ab3-high or ab3-low. This experiment was not presented in this thesis as it remains to be completed. The following hypotheses could be addressed in order to decipher the reason for the two types of responses from ab3A neurons.

Fruit detection was not impaired in the scope of this study. Yet, results of Chapter 6 comparing the responses with *D. melanogaster* also supports a change in function for ab3A as it was one of the most divergent ORN classes regarding the detection of ripe fruit volatiles. Or22a and Or22b are associated with ab3 in *D. suzukii* and a loss of function for

Or22a was described (Hickner *et al* 2016, Ramassamy *et al* 2015). Furthermore, in *D. melanogaster*, splices of functional or22a were identified (Shaw *et al.* 2019). These so-called chimera by Keesey *et al.* (2019) may be expressed in *D. suzukii*. How are these hypothetical splices only expressed in some flies? How are these expressed at the same time as other splices on a same animal? How are these genetic changes not affecting the function of ab3A in host selection?

All other ligands (activation over 100 impulses/s) for Or22a (tested with two dilutions of 1% and 0.01%) were not so different between the two lines, except for pentyl acetate, prenyl acetate and methyl hexanoate which activity was enhanced. It is sensible to consider that Or22a may thus not have been impaired. It can be hypothesised that two different ORs might be involved, one detecting β-cyclocitral (and perhaps being sensitive to the three other affected ligands) while the other detects the rest of the panel (Or22a). The roles of Or22a, Or22b and possibly unidentified ORs could be further explored with recordings of electrophysiological responses of single OR expression, as for *D. melanogaster*, using the "empty neuron" system (van der Goes van Naters and Carlson 2006; Mansourian *et al.* 2019).

The selection of ligands and ORN tested to compare the two lines was limited in this study hence, additional changes in the responses to chemicals may have been overlooked. However, it appears that very little changes occurred between the two lines, notably ab1A and ab4A responses to fruit volatiles did not vary. The two classes were also the most functionally divergent from *D. melanogaster* regarding the detection of ripe fruit odours (Chapter 6).

Lastly, some classes of OBPs are associated with the functional basiconic sensillum type ab3 and with the other compared, ab1 and ab4 (Table 1-1). It is unlikely that the two types of response to β-cyclocitral by ab3A are associated with changes in expression of OBPs because no changes in responses to fruit volatiles are observed that could be correlated with the expression of these OBPs.

8 GENERAL CONCLUSIONS

Adults *Drosophila suzukii* on a raspberry. Their leg crossing appears like a “high five”. ©Rothamsted Research

8 CONCLUSIONS AND FUTURE DIRECTIONS

The work presented in this thesis led to the description of a model of ripe fruit discrimination by the peripheral olfactory system in *Drosophila suzukii* for host selection and discrimination. The outcomes of this study provide novel information on the mechanism of host selection in *D. suzukii* and other polyphagous insects. This model was replicated in two host selection systems for *D. suzukii* (selection of fruits from taxonomically diverse plants, and selection of host fruits from closely related cultivars within one plant species) and was verified as a conserved mechanism from *Drosophila melanogaster*. It is a first study which looked at the encoding of whole fruit headspaces by the peripheral olfactory system, in addition to individual fruit volatiles, thereby recreating a most representative field situation as insects rarely detect individual compounds but mostly detect whole blends of chemicals from potential hosts.

Seven classes olfactory receptor neurons (ORNs) are dedicated to host selection and discrimination: pb1A, ab1A, ab2A, ab3A, ab4A and ab7A. These classes of ORNs are the most involved in ripe fruit detection in both species. Several combinations of these ORNs induce behavioural responses guiding the insect towards or away from ripe fruits. *D. suzukii* can discriminate each fruit type from a characteristic fruitprint on the olfactory system made by fruit-specific volatiles. Unlike for other ecologically relevant signalling, host selection is not governed by a single olfactory circuit: several ORNs are involved in multiple combinations of two or more classes of ORNs and none appeared to be necessary and sufficient.

This model enabled the fast identification of novel semiochemicals which can be developed for pest management. The identification of ORNs involved in attraction or aversion permits to rapidly test semiochemicals that are likely to induce a desired behavioural response. Furthermore, approximately 70 antennally active volatiles from ripe fruits are candidate semiochemicals to be behaviourally tested. The use of ripe fruit volatiles may be a novel alternative to overripe fruit volatiles which may be more specific to *D. suzukii*. They have a potential to be more attractive and thus more competitive with ripening fruits compared to fermenting volatiles.

8.1 HIGHLIGHTS

i. Combinatorial patterns of activation of ORNs in the peripheral olfactory system induce discrimination of ripe fruits suitable for oviposition in gravid female *D. suzukii*.
ii. A subset of seven out of 28 classes of ORNs is activated by ripe fruits from six taxonomically diverse plant species: pb1A, ab1A, ab2A, ab3A, ab4A, ab7A and abXA in both species.
iii. The classes pb1A, ab1A, ab3A and ab4A are associated with host shift in *D. suzukii*.
iv. The class ab3A is involved but does not drive host specialisation on ripe fruits on its own.
v. The classes pb1A and ab1A elicit attraction when simultaneously activated and were associated with attraction in chapters 3-6.
vi. The activation of ab7A was associated with non-attraction and masking of attractive odours in four independent studies, in combination with ab4A or ab2B.
vii. The skin thickness and ripeness stage of grapes (*Vitis vinifera)* appear to be detected via balanced activations of host activated pb1A and non-host activated ab4A and ab7A.
viii. The ab2B is associated with two distinct olfactory circuits: attraction in combination with ab3A and aversion to fruit volatiles in combination with ab7A.
ix. Ripe fruit detection was not impaired by a loss of sensitivity of ab3A to the volatile β-cyclocitral in *D. suzukii.*
x. *D. suzukii* and *D. melanogaster* differ in their detection of one enantiomer of nepetalactol. The patterns are distinct from the racemic mixture from parasitoid wasp mediating avoidance via ab10B in both species.
xi. Ripe fruit volatiles are candidate attractants and possible masking cues/repellents identified for pest management.

8.2 *FUTURE DIRECTIONS*

Behavioural studies are needed to advance the results of this study. How do the combinations of ORNs induce different behaviours? gradient behaviours (the least to the most attractive) could result from different combinations of ORNs. Different intensities of activation or the recruitment of additional ORNs may increase or decrease attractiveness. A threshold of activation above which behavioural responses are triggered was not fully characterised. From the 70 antennally active ripe fruit volatiles, candidate attractant may be validated with the common activation of pb1A, ab1A, ab2A and/or ab3A. Candidate masking cues or repellents may also be identified by their activation of ab7A, ab4A and/or abXA. Additional work to select the appropriate support to release chemicals in field trials and laboratory behavioural assays is needed for *D. suzukii*, which infestation rates urge for fast and improve novel management tools.

The characterisation of abX is further needed as one of the ORNs was involved in fruit detection. The roles of abY and ab9 ORNs are also unclear at this stage and require further characterisation. The discovery of a *D. suzukii* line (ab3-low) which ab3A ORNs did not respond to β-cyclocitral led to fundamental questions on the role of the volatile compound, which was claimed to drive specialisation to a novel ecological niche. This impairment did not affect *D. suzukii* ability to detect oviposition substrates. It remains unclear where this impairment came from and what could be made of it. Further molecular work will permit to determine whether mutations may have occurred and whether they can be used to develop novel *D. suzukii* genetic tools for its management. The use of repellent chemicals from wasps may be valuable tools but their detection mechanism in mixture might compromise the management effort. Additional study of nepetalactol enantiomers and their patterns of activation on the olfactory system may permit to identify the circuit mediating repellency. It may provide a possibility to manipulate predator-avoidance behaviour in order to either repel flies from crops or increase the control via natural predators via inhibition of their ability to sense predators. Furthermore, the study of how enantiomers of a same chemical are detected may inform on the mechanism of ligand-receptor binding which are currently unclear.

Lastly, evolutionary events in the *GR* lineages appeared having a secondary role in the host shift of the Suzukii subgroup behind *OR* gene lineages (Hickner *et al.* 2016). Their inclusion in future studies would bring further insights on the mechanisms of host selection. Similarly, ORNs in other structures (e.g. intermediate, ceoloconics) were not prioritised because there are no evidences to date of their role in long range fruit volatile detection in *Drosophila* (Dweck *et al.* 2018). Nonetheless, because host/food odours and fly odours were found to

be complementary to drive attraction in *D. melanogaster* (Reddy and Guerrero 2004; Duménil *et al.* 2016; Lebreton *et al.* 2017) their study could bring valuable insight.

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10 APPENDICES

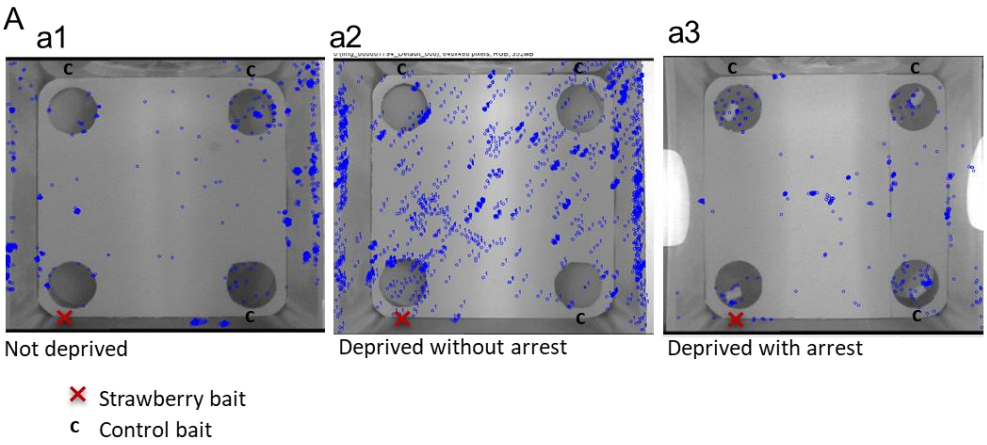
10.1 APPENDIX 1. OPTIMISATION OF BEHAVIOURAL ASSAYS

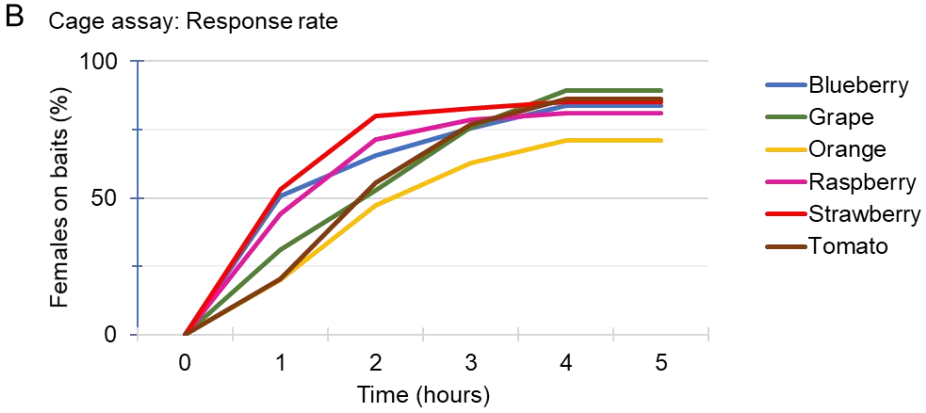
10.1.1 Optimisation of behavioural assays

Adult *Drosophila suzukii* are sitting around and on top of the food, rarely walking. Sometimes they fly from a spot to another and may have long stationary flights. They contrast with *Drosophila melanogaster*, which continuously walk around in the environment they are provided with. Their stationary behaviour made it challenging to find appropriate assays to

test their behavioural response to fruit and volatiles. Walking assays in the 4-arm olfactometer and Y-tube olfactometer were not optimal to test the behaviour *of D. suzukii* (Figure 10-2). Attempts were thus made to design or improve behavioural assays, which would allow to test the behavioural response of the flies to olfactory baits (whole fruit headspaces, chemical standards), with a response rate higher than one of the currently available tests (Figure 10-1).

Using a camera positioned above a 4-choice cage assay the position of 10 flies was monitored during 5 h with a picture every 2min. Attractive strawberry headspace was given as a bait and flies were tested after being either deprived or not. Pictures were analysed using Image J. Deprived flies were more active. This indicated that they may be in a foraging state, which is beneficial for the purpose of these tests. In addition, giving an arrest point on top of each bait containing sugar water permitted to keep the flies busy on top of the chosen bait, to be collected. It also ensured that behavioural responses related to food foraging was randomised and only a choice for oviposition would be observed. Therefore, a simple passage was not mistaken with the choice of resting and feeding on a given bait. The response rate in 4-choice cage assay and wind tunnel were monitored until all 100% flies tested reached the attractive upwind strawberry. It was concluded that after 5 h more than 80% of the females tested showed an attraction behaviour towards a host fruit.





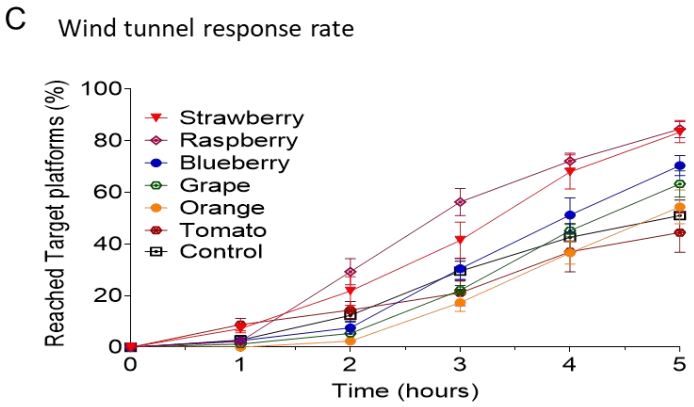


Figure 10-1 Optimisation of behavioural experimentation for D. suzukii

A) a1-a2. Effect of food and oviposition site deprivation on the overall activity of the flies in the 4- choices cage assay with strawberry as bait. a2-a3. Incorporation of an arrest made of sugar water enabled a focused attraction to the bait and controls, where females could be collected during the assay. B&C) Response rate over 5 h in a 4-choice cage assay (B, females collected from bait and controls) and a wind tunnel (C, females collected upwind on top of bait and control).

The walking response to olfactory cues was also tested using two types of test: Peterson 4-arm olfactometer and Y-tube olfactometer. The attractive fruits strawberries, raspberries and blueberries were used as baits against 1 or 3 controls which contained only water.

10.1.2 Design of the 4-arm Peterson olfactometer

A 115mm diameter (small) and a 150mm diameter (large) perspex olfactometers were constructed from three layers held together with plastic nuts and bolts. Filter paper (Whatman, type 1) was inserted between two layers to form a walking ground. Air was pumped through activated charcoal filter and humidified, then split between two glass chambers containing the bait and the control. The air stream left the chambers, carrying volatiles produced by the bait or control, into the arms of the olfactometer. Air was pulled out from the centre arena by another pump. Air flow meters ensured the airflow entering the arms was similar and that its total was a little higher than the air flow pulled out from the olfactometer. The created positive pressure ensured only the filtered air ran through the system. In order to remove visual stimuli from the bait, the chambers were hidden from the apparatus. The apparatus was washed with detergent and rinsed with 100% ethanol after 3 runs. A single female was inserted in the centre arena and observed for 20 min. Its position and time spent in each arm were recorded using specialist software (OLFA, Udine, Italy). The three control arms were regarded as one, to analyse the results. The centre of the apparatus was regarded as a “no choice” zone.

10.1.3 Design of the Y-tube olfactometer

A glass Y-tube (1cm diameter, 6.5cm arm length) was placed vertically under white light, and with an air entrainment system (described above). Glassware was washed with detergent, rinsed with 100% acetone and baked at 180°C at least 2 h, after 3 runs. Females were individually released into the main stem, where they encounter the two air streams. Each was observed for 10 min. First choice and time spent in each arm were recorded. Females staying in the main stem were regarded as “no choice”.

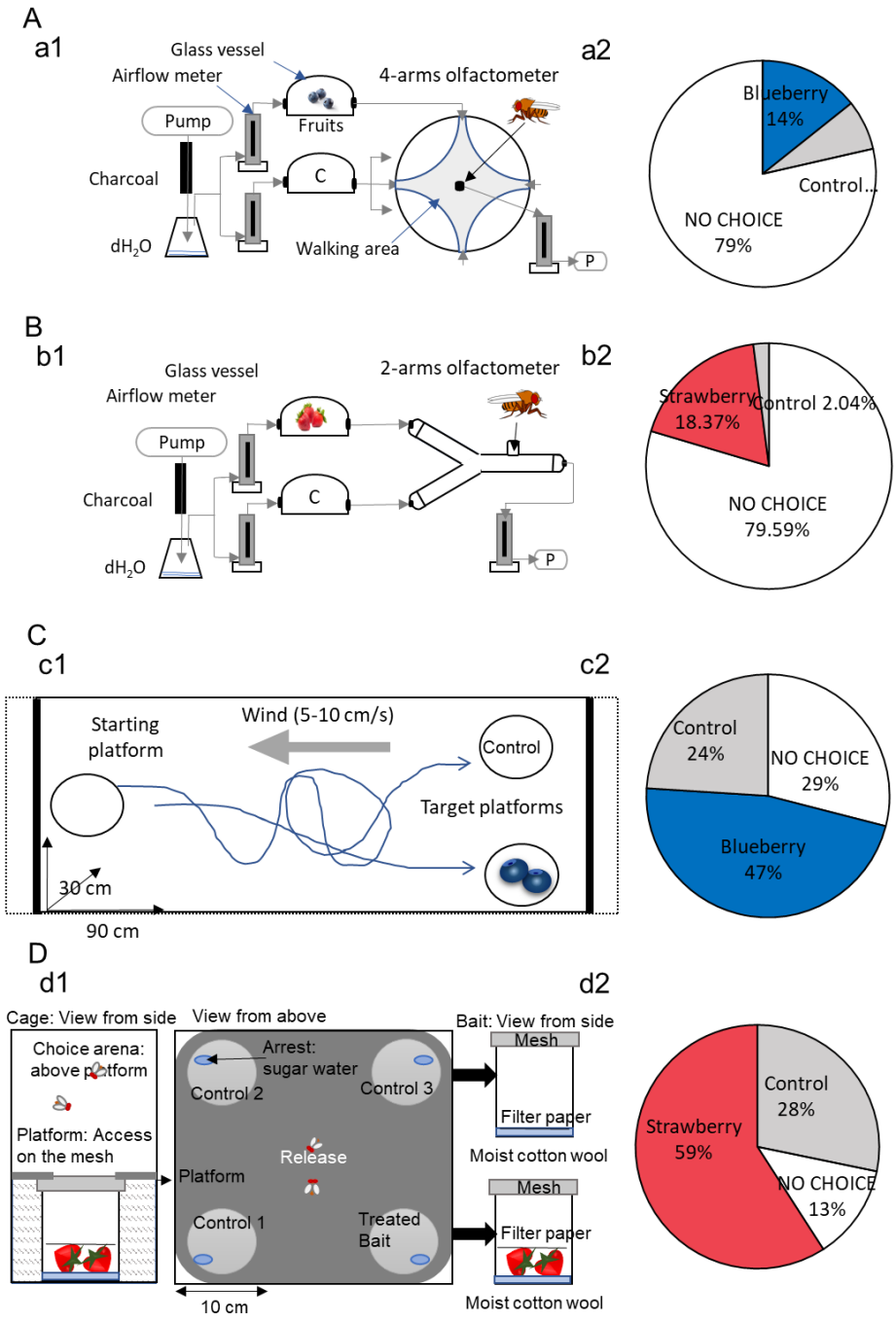
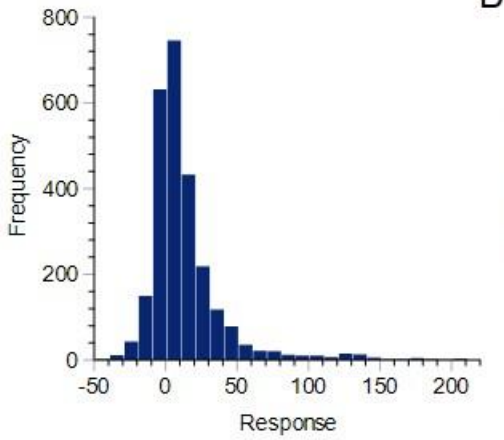


Figure 10-2 Behavioural responses of females D. suzukii in four different behavioural assays

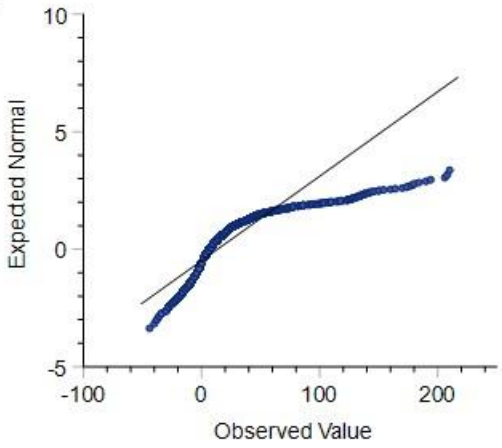
A) 4-arm olfactometer. a1) Scheme. a2) Percentage of females on blueberry, control (average of 3), no choice. Tests lasted 20 min with single females. B) Y-tube olfactometer. b1) Scheme. b2) Percentage of females on strawberry, control, no choice. Tests lasted 5-10 min with single females. C. Wind tunnel assay. c1) Scheme c2) Proportion of females on blueberry, the control, no choice. Test lasted 5h with 10-15 females. D) 4- choices cage assay. d1) Scheme. d2) Percentage of females on strawberry, control (average of the 3), no choice. Test lasted 5h, 10-15 females.

10.2 APPENDIX 2. ORN RESPONSES TO FRUIT HEADSPACES IN D. SUZUKII

A



B



C

	Statistic	df	P value		Statistic	df	P value
All responses	0.731	2600	<0.001	ab4A	0.782	104	<0.001
Per stimuli				ab4B	0.86	104	<0.001
Sp. act.	0.942	325	<0.001	ab5A	0.904	80	<0.001
Control	0.95	325	<0.001	ab5B	0.886	80	<0.001
Strawberry	0.799	325	<0.001	ab6A	0.977	80	0.168
Raspberry	0.805	325	<0.001	ab6B	0.944	80	0.002
Blueberry	0.816	325	<0.001	ab7A	0.934	104	<0.001
Grape	0.807	325	<0.001	ab7B	0.846	88	<0.001
Orange	0.651	325	<0.001	ab8A	0.873	96	<0.001
Tomato	0.784	325	<0.001	ab8B	0.911	96	<0.001
Per ORN class				ab10A	0.913	80	<0.001
ab1A	0.857	96	<0.001	ab10B	0.957	80	0.009
ab1B	0.904	96	<0.001	abXA	0.696	80	<0.001
ab1C	0.992	96	0.851	abXB	0.967	80	0.037
ab1D	0.981	96	0.165	pb1A	0.801	88	<0.001
ab2A	0.805	96	<0.001	pb1B	0.95	88	0.002
ab2B	0.973	96	0.044	pb2A	0.923	96	<0.001
ab3A	0.845	120	<0.001	pb2B	0.882	88	<0.001
ab3B	0.747	120	<0.001	pb3A	0.984	96	0.316
				pb3B	0.941	96	<0.001

Figure 10-3 Normality tests on ORN responses to fruit headspaces in D. suzukii

A) Distribution of data by frequency. B) QQ plot of measured data (dots) compared to an expected Gaussian distribution (line). C) Shapiro-Wilk normality test on the ORN responses to fruit headspaces. df: degree of freedom. P value <0.05 indicates that data do not follow a Gaussian distribution.

Table 10-1 ORNs responses to fruit headspace in D. suzukii

Mean (± SEM) number of impulses per second during a 0.5 s stimulus with headspaces from harvested whole ripe strawberries, raspberries, blueberries, grapes, oranges and tomatoes, a control (ambient air). The spontaneous activity (baseline) was deducted from the responses to control and headspaces.

	Strawberry	Raspberry	Blueberry	Grape	Orange	Tomato	Control	Baseline	N
ab1A	105 (± 14)	118 (± 9)	49 (± 11)	42 (± 4)	35 (± 4)	26 (± 4)	11 (± 4)	18 (± 3)	12
ab1B	15 (± 5)	37 (± 7)	21 (± 6)	15 (± 4)	9 (± 3)	4 (± 2)	7 (± 2)	20 (± 3)	12
ab1C	25 (± 6)	25 (± 4)	21 (± 3)	22 (± 3)	17 (± 4)	17 (± 3)	6 (± 2)	22 (± 2)	12
ab1D	-5 (± 3)	-7 (± 2)	2 (± 2)	5 (± 3)	5 (± 3)	0 (± 2)	1 (± 3)	10 (± 2)	12
ab2A	134 (± 14)	95 (± 9)	19 (± 8)	13 (± 5)	19 (± 8)	9 (± 3)	2 (± 2)	16 (± 2)	12
ab2B	-16 (± 4)	-25 (± 3)	-2 (± 4)	-2 (± 3)	-1 (± 4)	4 (± 3)	4 (± 2)	36 (± 2)	12
ab3A	121 (± 6)	50 (± 10)	34 (± 6)	10 (± 4)	45 (± 9)	30 (± 4)	12 (± 4)	22 (± 2)	15
ab3B	18 (± 4)	25 (± 5)	11 (± 3)	1 (± 3)	7 (± 4)	2 (± 3)	4 (± 4)	18 (± 2)	15
ab4A	20 (± 3)	23 (± 6)	15 (± 4)	73 (± 9)	6 (± 3)	45 (± 11)	2 (± 2)	15 (± 2)	13
ab4B	-1 (± 2)	-4 (± 2)	0 (± 1)	-3 (± 3)	5 (± 5)	-4 (± 2)	0 (± 2)	8 (± 2)	13
ab5A	16 (± 5)	4 (± 4)	2 (± 3)	3 (± 3)	10 (± 3)	1 (± 3)	2 (± 2)	9 (± 4)	10
ab5B	-1 (± 5)	-1 (± 5)	-2 (± 5)	2 (± 4)	2 (± 2)	1 (± 2)	3 (± 2)	33 (± 7)	10
ab6A	19 (± 3)	10 (± 4)	15 (± 5)	17 (± 3)	7 (± 4)	12 (± 4)	9 (± 3)	13 (± 3)	10
ab6B	4 (± 4)	0 (± 3)	0 (± 3)	0 (± 3)	-7 (± 4)	-3 (± 3)	-1 (± 2)	27 (± 5)	10
ab7A	30 (± 4)	28 (± 7)	14 (± 4)	18 (± 4)	17 (± 4)	74 (± 9)	3 (± 3)	18 (± 2)	12
ab7B	16 (± 5)	3 (± 4)	0 (± 3)	-1 (± 3)	1 (± 4)	20 (± 6)	4 (± 2)	14 (± 4)	12
ab8A	23 (± 5)	3 (± 3)	2 (± 2)	1 (± 2)	2 (± 2)	3 (± 2)	0 (± 2)	16 (± 3)	12
ab8B	21 (± 4)	3 (± 2)	2 (± 2)	3 (± 2)	-2 (± 1)	0 (± 2)	1 (± 2)	12 (± 3)	12
ab10A	6 (± 2)	2 (± 2)	3 (± 2)	-3 (± 3)	1 (± 3)	1 (± 4)	1 (± 3)	32 (± 5)	10
ab10B	12 (± 4)	3 (± 3)	0 (± 2)	24 (± 4)	3 (± 2)	31 (± 5)	8 (± 2)	20 (± 3)	10
abxA	104 (± 12)	16 (± 3)	18 (± 5)	7 (± 2)	113 (± 22)	11 (± 2)	0 (± 1)	8 (± 1)	10
abxB	-5 (± 3)	-1 (± 4)	1 (± 4)	-2 (± 2)	-2 (± 2)	-5 (± 2)	2 (± 3)	8 (± 2)	10
pb1A	89 (± 14)	37 (± 4)	75 (± 7)	7 (± 2)	11 (± 1)	6 (± 2)	2 (± 3)	15 (± 2)	11
pb1B	-10 (± 3)	-3 (± 3)	-10 (± 2)	-2 (± 2)	-3 (± 3)	0 (± 3)	2 (± 2)	23 (± 3)	11
pb2A	13 (± 4)	5 (± 5)	7 (± 5)	-1 (± 2)	3 (± 2)	5 (± 2)	0 (± 2)	20 (± 1)	11
pb2B	-2 (± 3)	0 (± 2)	-1 (± 2)	-3 (± 4)	-1 (± 5)	-3 (± 3)	1 (± 2)	32 (± 5)	11
pb3A	2 (± 2)	0 (± 3)	2 (± 2)	2 (± 1)	0 (± 2)	2 (± 2)	1 (± 2)	13 (± 2)	12
pb3B	16 (± 4)	2 (± 2)	2 (± 2)	0 (± 2)	-2 (± 2)	1 (± 2)	-1 (± 2)	12 (± 2)	12

Table 10-2 Statistical comparison of responses to fruit headspace and control by D. suzukii classes of ORNs

Wilcoxon signed ranked test between responses to fruit headspaces and their respective controls for each ORN type. ***P<0.001, **P<0.01, *P<0.05. N=10-15 on at least 3 females.

	Strawberry	Raspberry	Blueberry	Grape	Orange	Tomato
ab1A	Z= -4.14 P= <0.001***	Z= -4.16 P= <0.001***	Z= 3.26 P= <0.001***	Z= -3.83 P= <0.001***	Z= -3.13 P= <0.01**	Z= -2.29 P= 0.02*
ab1B	Z= -1.54 P= 0.13	Z= -3.36 P= <0.001***	Z= 1.97 P= 0.04*	Z= -1.34 P= 0.19	Z= -0.15 P= 0.89	Z= 0.99 P= 0.33
ab1C	Z= -2.41 P= 0.01*	Z= -3.48 P= <0.001***	Z= 3.27 P= <0.001***	Z= -3.02 P= <0.01**	Z= -2.61 P= <0.01**	Z= -2.85 P= <0.01**
ab1D	Z= 1.33 P= 0.19	Z= 2.58 P= <0.01**	Z= 0.72 P= 0.48	Z= -1.31 P= 0.19	Z= -0.79 P= 0.44	Z= 0.11 P= 0.92
ab2A	Z= -4.17 P= <0.001***	Z= -4.17 P= <0.001***	Z= 2.17 P= 0.02*	Z= -1.97 P= 0.04*	Z= -1.51 P= 0.13	Z= -1.92 P= 0.05
ab2B	Z= 2.95 P= <0.01**	Z= 4.17 P= <0.001***	Z= -0.88 P= 0.39	Z= 1.21 P= 0.23	Z= 0.61 P= 0.55	Z= -0.67 P= 0.51
ab3A	Z= -4.26 P= <0.001***	Z= -3.7 P= <0.001***	Z= 2.51 P= 0.01*	Z= 0.68 P= 0.5	Z= -2.5 P= 0.01*	Z= -2.33 P= 0.01*
ab3B	Z= -2.42 P= 0.01*	Z= -2.98 P= <0.01**	Z= 1.72 P= 0.08	Z= 0.35 P= 0.73	Z= -0.75 P= 0.46	Z= -0.03 P= 0.99
ab4A	Z= -3.84 P= <0.001***	Z= -3.22 P= <0.001***	Z= 2.62 P= <0.01**	Z= -4.34 P= <0.001***	Z= -1.32 P= 0.19	Z= -3.83 P= <0.001***
ab4B	Z= 0.41 P= 0.69	Z= 1.25 P= 0.22	Z= -0.14 P= 0.9	Z= 1.09 P= 0.28	Z= -0.68 P= 0.51	Z= 1.03 P= 0.31
ab5A	Z= -2.92 P= <0.01**	Z= -1.19 P= 0.24	Z= 0.84 P= 0.41	Z= -0.81 P= 0.44	Z= -2.14 P= 0.03*	Z= -0.2 P= 0.87
ab5B	Z= 0.49 P= 0.64	Z= 0.87 P= 0.4	Z= -1.26 P= 0.22	Z= 0.64 P= 0.53	Z= 0.76 P= 0.46	Z= 1.3 P= 0.2
ab6A	Z= -2.07 P= 0.03*	Z= 0 P= 1	Z= 0.64 P= 0.53	Z= -1.98 P= 0.04*	Z= -0.23 P= 0.83	Z= -0.54 P= 0.61
ab6B	Z= -1.07 P= 0.3	Z= -0.12 P= 0.92	Z= -0.08 P= 0.95	Z= -0.23 P= 0.83	Z= 1.02 P= 0.32	Z= 0.45 P= 0.66
ab7A	Z= -3.65 P= <0.001***	Z= -1.37 P= 0.18	Z= 2.05 P= 0.03*	Z= -1.95 P= 0.05	Z= -2.38 P= 0.01*	Z= -3.6 P= <0.001***
ab7B	Z= -2.12 P= 0.03*	Z= -2.99 P= <0.01**	Z= 0.23 P= 0.83	Z= -0.88 P= 0.39	Z= 0.64 P= 0.54	Z= -2.19 P= 0.02*
ab8A	Z= -3.3 P= <0.001***	Z= -0.21 P= 0.85	Z= 0.52 P= 0.61	Z= -0.32 P= 0.76	Z= -0.5 P= 0.63	Z= -0.71 P= 0.5
ab8B	Z= -3.4 P= <0.001***	Z= -0.03 P= 0.98	Z= -0.53 P= 0.61	Z= -0.09 P= 0.94	Z= 1.57 P= 0.12	Z= 0.72 P= 0.48
ab10A	Z= -1.11 P= 0.28	Z= 0.22 P= 0.83	Z= 0 P= 1	Z= 1.36 P= 0.18	Z= 0.49 P= 0.64	Z= 0.07 P= 0.95
ab10B	Z= -0.58 P= 0.58	Z= 1.22 P= 0.23	Z= -2.21 P= 0.02*	Z= -2.85 P= <0.01**	Z= 1.32 P= 0.19	Z= -2.88 P= <0.01**
abXA	Z= -3.8 P= <0.001***	Z= -3.42 P= <0.001***	Z= 2.81 P= <0.01**	Z= -2.38 P= 0.01*	Z= -3.8 P= <0.001***	Z= -3.09 P= <0.01**
abXB	Z= 1.63 P= 0.1	Z= 1.64 P= 0.1	Z= 0.3 P= 0.78	Z= 1.56 P= 0.12	Z= 1.14 P= 0.26	Z= 1.98 P= 0.04*
pb1A	Z= -3.99 P= <0.001***	Z= -3.79 P= <0.001***	Z= 3.98 P= <0.001***	Z= -2.07 P= 0.03*	Z= -3.05 P= <0.01**	Z= -1.89 P= 0.06
pb1B	Z= 3.07 P= <0.01**	Z= 1.48 P= 0.14	Z= -3.21 P= <0.001***	Z= 1.29 P= 0.2	Z= 1.55 P= 0.12	Z= 0.03 P= 0.98
pb2A	Z= -2.45 P= 0.01*	Z= -0.2 P= 0.85	Z= 0.62 P= 0.54	Z= 0.53 P= 0.61	Z= -0.9 P= 0.38	Z= -1.99 P= 0.04*
pb2B	Z= 0.23 P= 0.83	Z= 0.53 P= 0.61	Z= -0.64 P= 0.54	Z= 0.76 P= 0.46	Z= -0.27 P= 0.8	Z= 0.79 P= 0.44
pb3A	Z= -0.27 P= 0.8	Z= 0.52 P= 0.61	Z= 0.58 P= 0.57	Z= -0.12 P= 0.91	Z= 0.49 P= 0.63	Z= -0.24 P= 0.83
pb3B	Z= -3.19 P= <0.001***	Z= -1.17 P= 0.25	Z= 0.81 P= 0.43	Z= -0.56 P= 0.59	Z= 0.72 P= 0.48	Z= -0.79 P= 0.44

10.3 APPENDIX 3. ORN RESPONSES TO HEADSPACES OF DIFFERENT CULTIVARS OF STRAWBERRY AND BLUEBERRY.

Table 10-3 Responses to headspaces of different fruit cultivars in D. suzukii

Mean responses rates (± SEM) during a 0.5 s stimulus with control (ambient air) and headspaces of 3 cultivars of strawberry and blueberry fruits.

	Spontaneous activity	Control	Strawberry			Blueberry			N
			Cuna	Sabrina	WinterStar	Biloxi	Legacy	Ventura	
ab1A	13.33 (± 2.71)	10 (± 2.3)	170.66 (± 8.77)	141.66 (± 10.21)	113.66 (± 16.99)	63.66 (± 9.34)	65.33 (± 10.74)	32.66 (± 7.87)	6
ab1B	12.33 (± 1.49)	7.66 (± 3.59)	45 (± 6.16)	35 (± 10.52)	26.66 (± 7.33)	11.33 (± 2.1)	12.66 (± 3.41)	1.66 (± 0.95)	6
ab1C	20.33 (± 3.32)	12.66 (± 6.82)	26.33 (± 9.42)	24.66 (± 7.13)	13.66 (± 6.74)	23.33 (± 5.02)	25.66 (± 5.85)	6 (± 5.68)	6
ab1D	12 (± 3.05)	1.33 (± 1.52)	-8 (± 4.35)	-8.34 (± 4.07)	-7.34 (± 4.21)	-4 (± 4.19)	-6 (± 3.54)	-3.34 (± 3.45)	6
ab2A	10.33 (± 3.36)	3.33 (± 1.42)	134.33 (± 9.99)	122.66 (± 10.54)	69 (± 9.4)	26.33 (± 5.87)	26.33 (± 4.39)	7.33 (± 6.46)	6
ab2B	26 (± 6.42)	1 (± 1.69)	-5.34 (± 5.5)	-8.67 (± 7.91)	19 (± 12.45)	-8.34 (± 6.72)	-8 (± 5.08)	3.33 (± 5.81)	6
ab3A	18 (± 2.92)	6 (± 2.63)	125.33 (± 10.88)	117.33 (± 14.1)	126 (± 14.28)	43 (± 7.31)	39.66 (± 8.12)	7.33 (± 4.05)	6
ab3B	9 (± 2.72)	1.33 (± 3.6)	61.66 (± 12.34)	42 (± 10.05)	52 (± 15.78)	23.33 (± 4.34)	10 (± 4.35)	5.33 (± 3.04)	6
ab4A	13.66 (± 2.15)	1.33 (± 4.75)	74.66 (± 4.91)	53.66 (± 13.5)	35 (± 5.67)	5.33 (± 2.66)	4.33 (± 1.96)	11.33 (± 6.35)	6
ab4B	7.66 (± 1.74)	2 (± 2.06)	1.66 (± 1.49)	2.66 (± 4.24)	5 (± 1.84)	1.66 (± 2.38)	-0.34 (± 2.21)	0.33 (± 2.98)	6
ab5A	1 (± 0.68)	0.33 (± 0.8)	55.66 (± 8.63)	46.66 (± 7.05)	55.66 (± 8.72)	2 (± 1.71)	2.66 (± 3.48)	1 (± 0.85)	6
ab5B	24.66 (± 4.58)	28.66 (± 10.59)	14.66 (± 11.65)	-2 (± 5.95)	12.66 (± 10.34)	20.33 (± 9.72)	15.66 (± 10.13)	18 (± 8.8)	6
ab6A	12 (± 3.74)	6 (± 4.69)	24.5 (± 3.2)	13.5 (± 7.22)	15 (± 6.6)	8 (± 4.08)	7.5 (± 4.57)	6.5 (± 5.73)	4
ab6B	14.5 (± 6.75)	2 (± 0.81)	17.5 (± 5.9)	21.5 (± 8.3)	20 (± 12.67)	17.5 (± 3.3)	12 (± 2.7)	2.5 (± 2.62)	4
ab7A	14 (± 4.67)	5.66 (± 3.63)	38 (± 10.75)	27.33 (± 11.35)	26.66 (± 9.98)	11.33 (± 5.48)	16 (± 5.56)	16.66 (± 7.97)	6
ab7B	25 (± 7.51)	7.33 (± 4.86)	25 (± 6.21)	8 (± 3.75)	7.33 (± 3.52)	-5.67 (± 6.93)	-7.34 (± 5.43)	-3.67 (± 6.29)	6
ab8A	8.66 (± 2.61)	2.33 (± 0.8)	73 (± 20.98)	24.33 (± 5.87)	20.66 (± 8.8)	2 (± 2.92)	3 (± 1.52)	2.66 (± 3.37)	6
ab8B	10 (± 3.14)	1.33 (± 2.29)	43.66 (± 11.81)	11 (± 6.25)	8.66 (± 7.24)	7 (± 3.04)	3.33 (± 3.08)	2.66 (± 1.52)	6
ab10A	6 (± 0.89)	1.33 (± 2.17)	91 (± 9.86)	97 (± 12.77)	56 (± 7.41)	13 (± 2.81)	3.33 (± 2.23)	2.66 (± 2.66)	6
ab10B	7.33 (± 2.17)	-2.34 (± 3.11)	-2.34 (± 3.11)	-2.34 (± 2.6)	-2.34 (± 2.33)	1 (± 3.95)	0.66 (± 1.6)	0 (± 3.38)	6

Table 10-4 Statistical comparison of the responses to headspaces of different fruit cultivars

ORNs for which a Kruskall-Wallis test and a Mann-Whitney Bonferroni corrected comparison were significant. Cultivars were tested on the same individual ORNs. N= 6 replicates.

	Chi2	P value	Pairs	Z value	P value*
Blueberry cultivars					
ab3A	9.031	0.010	Legacy: Ventura	2.566	0.019
			Biloxi: Ventura	2.580	0.025
ab1B	10.87	0.004	Legacy: Ventura	2.746	0.019
			Biloxi: Ventura	2.897	0.006
ab3B	6.364	0.041	Biloxi: Ventura	2.406	0.038
Strawberry cultivars					
ab1A	8.274	0.015	Winterstar: Cuna	2.562	0.025
ab4A	9.480	0.008	Winterstar: Cuna	2.892	0.006
ab2A	9.950	0.006	Winterstar: Sabrina	2.571	0.019
			Winterstar: Cuna	2.566	0.025

*Adjusted *P* value after Bonferroni multiple comparison correction.

10.4 APPENDIX 4. BEHAVIOURAL RESPONSES TO FRUIT HEADSPACES BY D. SUZUKII

Table 10-5 The 4-choice cage assay with fruit headspaces

4-Choice cage assay with fruit headspaces. Mean ±SEM proportion of females that: chose the treatment, chose one of the controls, are found above and under the platform (No choice). N is the number of replications. Response is the proportion of females which chose one of the baits within the 5 hours trial. GLM Poisson distribution. Comparison of treatment and control (all 3 pooled together).

	Raspberry	Strawberry	Blueberry	Grape	Orange	Tomato	Control
Mean +/-SEM							
Compounds	**5.1(±0.6)**	**4.1(±0.6)**	**3.2(±0.5)**	2.6(±0.6)	2(±0.6)	2.7(±0.9)	3.5(±0.6)
control1	**1.9(±0.4)**	2.4(±0.5)	2.5(±0.5)	2.6(±0.4)	1.5(±0.3)	2.9(±0.6)	2.7(±0.6)
control2	2.2(±0.5)	1.6(±0.2)	2.4(±0.5)	2.6(±0.5)	2.6(±0.8)	2(±0.8)	3.6(±0.6)
control3	2.3(±1.1)	1.7(±0.3)	2.9(±0.6)	2.2(±0.6)	1.7(±0.4)	2.5(±0.6)	2.3(±0.6)
nochoice	1.1(±0.5)	1.6(±0.5)	2.2(±0.6)	1.3(±0.4)	2.8(±1)	1.6(±0.5)	2.5(±0.5)
Resp.%	**87(±11)**	**85(±16)**	**85(±19)**	89(±18)	69(±22)	86(±18)	88(±0)
Nrepl.	10	10	14	10	10	10	10
Nfem./Repl.	7-19	9-16	6-19	8-14	8-16	9-17	11-17
Control1 vs 2:							
Z-test	**0.468**	-1.25	-0.25	0	1.696	-1.278	1.13
P value	0.639	0.21	0.81	1	0.089	0.201	0.258
Control1 vs 3:							
Z-test	0.616	-1.09	0.58	-0.577	0.353	-0.544	-0.565
P value	0.537	0.28	0.56	0.564	0.723	0.587	0.572
Control2 vs 3:							
Z-test	**0.149**	**0.174**	**0.818**	-0.577	-1.362	0.744	-1.678
P value	0.881	0.861	0.413	0.564	0.173	0.456	0.093
Bait vs Control							
test	-4.643	-3.756	-1.258	-0.231	0.131	-0.402	-0.996
P value	<0.001	<0.001	0.209	0.817	0.896	0.688	0.319
Bait vs noChoice							
Bait vs noChoice	-4.902	-4.119	-4.502	-3.082	-0.493	-2.337	-1.996
P value	<0.001	<0.001	<0.001	0.002	0.622	0.019	0.046

Table 10-6 The wind tunnel assay with fruit headspaces: summary and statistics

Mean (+/- SEM) number of females which reached the target platforms (upwind attraction) and which chose the fruit over the water (Choice) during 5hrs wind tunnel assay. "%Resp.", proportion of females collected upwind (Responders).

	Strawberry	Raspberry	Blueberry	Grape	Orange	Tomato	Control (Water)
%Resp.	83 (± 2)	84 (± 1)	70 (± 2)	63 (± 2)	54 (± 3)	44 (± 5)	51 (± 4)
Nrepl.	7	6	6	6	6	6	8
N*	22-50	19-43	21-40	25-37	20-31	12-21	6-29
Upwind attraction							
Nupwind	29(±4)	23(±2)	22(±3)	19(±1)	14(±2)	7(±1)	10(±2)
Ndownwind	5(±1)	5(±2)	9(±1)	12(±2)	11(±1)	8(±2)	9(±1)
t value[1]	-4.772	-3.964	-2.295	-1.104	-0.15	1.158	
P value[1]	<0.001	<0.001	0.0273	0.2766	0.8812	0.2542	
PI-upwind	0.67(±0.08)	0.69(±0.07)	0.41(±0.08)	0.27(±0.1)	0.09(±0.13)	-0.11(±0.15)	0.02(±0.12)
t-test	8.12	10.25	5.227	2.652	0.673	0.721	0.167
P value	<0.001	<0.001	0.003	0.045	0.531	0.503	0.872
upwind Distribution							
Nbait	25(±4)	19(±1)	15(±2)	13(±2)	8(±2)	5(±1)	6(±1)
N water	4(±1)	4(±1)	7(±1)	6(±2)	6(±1)	2(±1)	4(±1)
Fruit vs Control							
t value[2]	4.485	3.401	0.983	1.337	-0.384	1.28	
P value[2]	<0.001	<0.001	0.326	0.181	0.701	0.201	
Bait vs Water							
Nf, Nw[3]	175, 31	112, 26	87, 44	80, 36	46, 35	28, 11	49, 33
P value[3]	<0.001	<0.001	<0.001	<0.001	0.266	<0.01	0.097
PI-Choice	0.7(±0.05)	0.65(±0.08)	0.32(±0.09)	0.38(±0.15)	0.05(±0.15)	0.51(±0.15)	0.07(±0.18)
t-test	14.63	8.227	3.545	2.5	0.336	3.373	0.378
P value	<0.001	<0.001	0.016	0.054	0.751	0.019	0.716

*N females per N replication (Nrepl.). [1]Quasibinomial GLM on the distribution of females (upwind, downwind) in fruit experiments versus Control [Residual deviance: 72.671 on 38 degrees of freedom]; [2]Binomial GLM on the distribution of females on the two baits, fruit experiments versus Control [Residual deviance: 52.446 on 38 Degrees of freedom]. [3]Binomial test on the number of females collected on the fruit bait (Nf) and the water bait (Nw), within each experiment.

Table 10-7 The multiple-choice assay with three different fruits after exposure: summary and statistics

GLM with Poisson distribution. Numbers of females collected on fruits were compared to the number collected on the Control (water), for each rearing. Pv: Percentage of total variation. DF: degrees of freedom.

Source of Variation	Pv	DF	F test	*P value*
choice x rearing	2.59	9	1.031	0.421
choice	67.66	3	80.69	<0.001
rearing	0.09	3	1.003	0.403
Subject	1.04	34	0.1091	0.999
Residual		102		

Exposure	Raspberry	Blueberry	Grape	Control	No choice	N
Raspberry						
Mean ±SEM	6.4 (±0.9)	2.9 (±0.7)	1.5 (±0.2)	0.5 (±0.2)	1.1 (±0.4)	11
z-value	5.776	3.763	2.049			
P value	<0.001	<0.001	0.046			
Blueberry						
Mean ±SEM	6.1 (±0.6)	2.4 (±0.5)	2 (±0.4)	0.9 (±0.3)	0.8 (±0.4)	10
z-value	5.359	2.509	1.989			
P value	<0.001	0.01	0.046			
Grape						
Mean ±SEM	7.2 (±1)	2.8 (±0.7)	0.7 (±0.3)	0.1 (±0.1)	0.6 (±0.5)	9
z-value	4.143	3.156	1.659			
P value	<0.001	0.001	0.097			
Media						
Mean ±SEM	5.8 (±0.7)	2.2 (±0.7)	1.3 (±0.3)	0.3 (±0.2)	0.9 (±0.4)	9
z-value	4.804	3.064	2.148			
P value	<0.001	0.002	0.031			

10.5 APPENDIX 5. VOLATILES FROM FRUIT HEADSPACE EXTRACTS.

Table 10-8 Antennally active volatiles in harvested fruit headspace extracts

Antennally active compounds as identified from GC-EAG on antenna from gravid female *D. suzukii*. KI, kovat indices of antennally active peaks from the GC-EAG recordings and from tentative identification with GC-MS. For each extract, annotations (left column) are referring to graphs in Figure 4-1.

	Compound	KI_{GC-EAG}	KI_{GC-MS}	Chemical class	CAS
Strawberry					
1	Methyl butanoate	712	712	Ester	623-42-7
2	Isoamyl alcohol	724	724	Alcohol	123-51-3
3	Isobutyl acetate	765	760	Ester	110-19-1
4	n-Butyric acid	779	774	Acid	107-92-6
5	Ethyl butanoate	788	786	Ester	105-54-4
6	Butyl acetate	798	797	Ester	123-86-4
7	Methyl pentanoate	810	808	Ester	624-24-8
8	Isopropyl butanoate	832	830	Ester	638-11-9
9	Isoamyl acetate	864	862	Ester	123-92-2
10	Pentyl acetate	897	896	Ester	628-63-7
11	Prenyl acetate	903	903	Ester	1191-16-8
12	Methyl hexanoate	909	910	Ester	106-70-7
13	Sec-butyl butanoate	926	926	Ester	819-97-6
14	Methyl (E)-2-hexenoate	947	946	Ester	22210-20-4
15	Hexanoic acid	967	966	Acid	142-62-1
16	Hexyl acetate	995	996	Ester	142-92-7
17	(S)-Linalool	1086	1088	Terpene alcohol	126-90-9
18	Benzyl acetate	1138	1136	Ester	140-11-4
19	4-Decalactone	1437	1434	Cyclic ester	706-14-9
Raspberry					
1	Propyl acetate	700	700	Ester	109-60-4
2	Isobutyl acetate	764	760	Ester	110-19-0
3	Butyl acetate	798	797	Ester	123-86-4
4	1-Hexanol	856	854	Alcohol	111-70-6
5	2-Methylbutyl acetate	866	864	Ester	624-41-9
6	4-Penten-1-yl acetate	869	867	Ester	1576-85-8
7	2-Heptanone	872	870	Ketone	110-43-0
8	Prenyl acetate	901	903	Ester	1191-16-8
9	6-Methyl-5-hepten-2-one	959	965	Ketone	110-93-0
10	Myrcene	978	984	Terpene	123-35-3
Blueberry					
1	Ethyl isobutanoate	760	749	Ester	97-62-1
2	Methyl isopentanoate	773	765	Ester	556-24-1
3	Ethyl isopentanoate	846	842	Ester	108-64-5
4	1-Hexanol	860	857	Alcohol	111-70-6

5	Isopropyl pentanoate	885	883	Ester	NA
6	Ethyl 3-methyl-2-butenoate	907	905	Ester	NA
7	6-Methyl-5-hepten-2-one	967	965	Ketone	110-93-0
8	(*S*)-Linalool	1085	1088	Terpene alcohol	126-90-9
9	4-Ethylacetophenone	1241	1238	Aromatic ketone	937-30-4
Grape					
1	(*E*)- 2-Hexenal	835	830	aldehyde	505-57-7
2	1-Hexanol	860	857	Alcohol	111-70-6
3	6-Methyl-5-hepten-2-one	967	965	Ketone	110-93-0
4	Octanal	987	983	aldehyde	124-13-0
5	*(S)*-Linalool	1085	1085	Terpene alcohol	126-90-9
Orange					
1	Butyl acetate	798	797	Ester	123-86-4
2	1-Hexanol	857	857	Alcohol	111-70-6
3	Methyl hexanoate	907	907	Ester	106-70-7
4	1-Heptanol	956	957	Alcohol	111-70-6
5	6-Methyl-5-hepten-2-one	966	965	Ketone	110-93-0
6	Butyl butanoate	980	980	Ester	109-21-7
7	Hexyl acetate	994	995	Ester	142-92-7
8	(*R*)- Limonene	1031	1024	Cyclic terpene	5989-27-5
9	*(E)*-Ocimene	1041	1041	Terpene	3779-61-1
10	1-Octanol	1055	1056	Alcohol	111-87-5
11	(*S*)-Linalool	1087	1085	Terpene alcohol	126-90-9
12	DMNT*	1108	1106	Terpene	19945-61-0
13	Hexyl butanoate	1174	1175	Ester	2639-63-6
14	β- Cyclocitral	1206	1198	Cyclic aldehyde	432-25-7
15	Hexyl hexanoate	1370	1373	Ester	6378-65-0
Tomato					
1	Isobutyl acetate	766	760	Ester	110-19-2
2	(*Z*)-3-Hexenal	780	776	Aldehyde	6789-80-6
3	(*E*)- 2-Hexenal	826	832	Aldehyde	505-57-7
4	(Z)-3-Hexen-1-ol	843	845	Alcohol	928-96-1
5	Nitropentane	873	869	Nitroalkane	628-05-7
6	α-Pinene	928	928	Terpene	80-56-8
7	6-Methyl-5-hepten-2-one	969	965	Ketone	110-93-0
8	6-Methyl-5-hepten-2-ol	981	979	Alcohol	1569-60-4
9	2-Isobutylthiazole	1021	1017	Thiazole	18640-74-9

*(*E*)-4,8-Dimethyl-1,3,7-nonatriene.

Table 10-9 Additional volatiles from headspaces of harvested and non-harvested fruits.

Headspace extract from harvested fruit (HF) and whole plant, including non-harvested fruits and leaves (WP). Extracts were analysed on cool on column (COC)-GC and Optic column (OC)-GC. Kovat index (KI) from antennally active peaks of GC-EAG. Tentative identification with GC-MS. “No id.” No compounds matched the peak KI. EAG on standards: tentatively identified compounds were not active when tested alone with EAG.

Extract	GC*	KI	GC-EAG	Tentative identification	EAG
strawberry					
HF	COC	1124	active	No id.	
HF	COC	1182	active	Estrogole or Decanal	not active
HF	COC	1340	active	methyl 3 phenyl propenoate	not active
HF	COC	1554	active	(*E*)-nerolidol	not active
HF	OC			acetic acid	
HF	OC			methyl acetate	active
HF	OC			ethyl acetate	active
HF	OC			isopropyl actetate	
HF	OC			2,3-butanedione	
WP	OC			Methyl-2-oxo-propanoate	
L	OC			2 methyl-2-propenol	
WP	OC			2-butanone	active
HF	OC			(*E*)-methyl-2-butenoate	
Raspberry					
HF	COC			(E)-1,3- butadienol	
HF	COC			5-Hepten-2-one	
HF	COC	888	active	No id.	
HF	COC	1028	active	Beta-phellandrene	not active
HF	COC	1462	active	(*E*)-Beta-farnesene	not active
HF	COC/ OC			4-Hydroxy-2-butanone	active
HF	COC			Isoamyl acetate	active
HF	OC			acetic acid	
WP	OC			ethyl acetate	active
WP	OC			2methyl-2-propenol	
HF	OC			2,3-butanediol	
HF	OC			2-ethyl-2-butenal	
HF	OC			2 or 3-methylfurane	
Blueberry					
HF	COC		active	No id.	
HF	COC	735	active	No id.	
HF	COC	803	active	No id.	
WP	OC			methyl cyclopentane	
HF	OC			heptane	
HF	OC			2-pentenol	
L	OC			acetic acid	
WP	OC			2,3-butanediol	
WP	OC			3-methyl-1-butanol	

Grape					
HF	COC	739	active	No id.	
HF	COC	806	active	No id.	
HF	COC	868	active	No id.	
HF	COC	1024	active	No id.	
HF	COC	1349	active	No id.	
HF	COC	1535	active	No id.	
HF	COC	1100	active	undecane	not active
HF	COC	1199	active	dodecane	not active
Orange					
HF	COC	900	active	Nonane	not active
HF	COC	931	active	4-Methyl-1-pentanol	not active
HF	COC	1439	active	(E)-Caryophyllene	not active
Tomato					
HF	COC	826-831	active	(Z)-2-Hexenal	
HF	COC	890	active	No id.	
HF	COC	1187	active	Decanal	not active
HF	COC	1737	active	No id.	
WP	OC			3 methyl 1 butanol	
WP	OC			2 methyl 1 butanol	
WP	OC			butyl acetate	active
WP	OC			isopropyl actetate	active

10.6 APPENDIX 6. BEHAVIOURAL RESPONSES TO FRUIT VOLATILES IN D. SUZUKII

Table 10-10 The 4-choice assay with fruits and volatiles: summary and statistics

Numbers of females collected on each bait and in the cage (no choice) during a 5 h 4-choice cage assay. GLM with Poisson distribution comparing the numbers of females on the bait with the numbers on controls and no choice. All compounds were in 1% solution with paraffin oil. Paraffin oil was used as a control stimuli. "Car" Cardiff, "Rres" Rothamsted.* Both data were pooled together. 1/2S: the dose of S-Linalool used was the same as in the RS mixture. "sSu" small and "lSu" large capillaries with 6-methyl-5-hepten-2-one. "3R" and 5R", three and five raspberries. "Repl." Replication.

	MP	MP-sSu	S +PO	S+sSu	S+lSu	3R	3R-1T	5R-1T
Mean +/-SEM								
Compounds	3.8(±2.1)	2.9(±1.4)	0(±1.7)	2.4(±1.1)	2.8(±1.7)	4.7(±2.8)	3.8(±1.9)	4(±0.7)
control1	2.3(±1.6)	2.6(±1.4)	0(±0.9)	4(±2.1)	2(±1.2)	2.7(±1.9)	3.1(±1.4)	2.6(±1.9)
control2	2.7(±1.9)	1.8(±1.4)	0(±1.3)	2.6(±1.5)	2.3(±1.7)	2.6(±2.5)	3.1(±2)	0.8(±1.1)
control3	2.3(±0.8)	2.3(±1.4)	0(±1.8)	4.6(±2.9)	2.7(±2.1)	3.8(±1.8)	2.5(±1.2)	0.4(±0.5)
nochoice	1.5(±0.6)	3.5(±0.8)	0(±0)	0.8(±0.4)	2.6(±0.8)	0.4(±0.2)	0.8(±0.4)	2(±0.6)
Resp.%	89(±16)	75(±14)	81 (±17)	94 (±16)	79 (±17)	93 (±12)	98 (±20)	86 (±22)
Nrepl.	10	10	10	5	10	20	10	10
Nfem./Repl.	9-17	11-16	12-20	10-15	10-17	8-16	9-17	9-17
Control1 vs 2:								
Z-test	0.565	-1.199	-0.267	1.399	-1.149	-0.137	0	-2.061
P value	0.572	0.23	0.789	0.162	0.25	0.891	1	0.039
Control1 vs 3:								
Z-test	0	-0.428	0.13	0.2	-0.699	1.358	-1.088	-2.464
P value	1	0.668	0.896	0.841	0.485	0.175	0.276	0.014
Control2 vs 3:								
Z-test	0.565	0.779	0.397	-1.209	0.457	1.491	-1.089	-0.8
P value	0.572	0.436	0.691	0.227	0.647	0.136	0.276	0.423
Bait vs Control								
Z-test	-2.228	-1.175	-1.298	-1.668	-0.583	-2.438	-1.679	-3.589
P value	0.026	0.239	0.194	0.09	0.56	0.015	0.093	<0.001
Bait vs noChoice								
Z-test	-3.338	1.946	-0.982	-3.318	-0.127	-3.811	-4.971	-2.773
P value	<0.001	0.051	0.326	<0.001	0.891	<0.001	<0.001	0.005
Bait composition								
	Methyl propionate	Methyl propionate 6-methyl-5-hepten-2-one	Strawberry	Strawberry 6-methyl-5-hepten-2-one	Strawberry 6-methyl-5-hepten-2-one	Raspberry	Raspberry Tomato	Raspberry Tomato

	Bait-S Car	Bait-S Rres	Bait-S*	Bait-1/2S	Bait-RS	(S)-Linalool	β-Cyclocitral	Ethyl acetate
Mean +/-SEM								
Compounds	3.8(±3)	4.7(±2.6)	4.3(±2.8)	3.2(±1.6)	1.5(±1)	2.9(±1.7)	2.6(±1.5)	2.5(±1.3)
control1	1.9(±1.7)	2.2(±1.9)	2.1(±1.8)	3.4(±2)	2.6(±1.6)	1.7(±1.8)	3(±2.1)	2.7(±1.6)
control2	1.8(±2)	1.8(±2)	1.8(±2)	2.7(±1.8)	1.9(±0.9)	2.9(±1.7)	2(±1.3)	2.9(±1.4)
control3	2.7(±2.2)	3.2(±2.1)	3(±2.1)	2.2(±2.1)	2.2(±1.1)	1.8(±1.5)	2.7(±1.8)	2.8(±2.1)
nochoice	1.9(±0.6)	0.6(±0.4)	1.3(±0.5)	0.9(±0.4)	2.4(±0.7)	2.4(±0.7)	2.5(±0.8)	1.8(±0.8)
Resp.%	96(±21)	83(±21)	90 (±22)	93(±20)	78(±13)	79(±17)	80(±16)	90(±17)
Nrepl.	10	10	20	10	10	10	10	10
Nfem./Repl.	10-17	9-16	9-17	10-15	7-13	8-17	10-16	10-17
Control1 vs 2:								
Z-test	2.798	-0.631	-0.569	-0.894	-1.039	0.169	-1.405	0.267
P value	0.005	0.527	0.569	0.371	0.299	0.865	0.16	0.789
Control1 vs 3:								
Z-test	1.173	1.353	1.79	-1.591	-0.577	1.748	-0.397	0.35
P value	0.24	0.176	0.073	0.112	0.564	0.08	0.691	0.893
Control2 vs 3:								
Z-test	1.332	1.953	2.336	-0.713	0.468	-1.589	1.017	-0.132
P value	0.18	0.051	0.019	0.476	0.639	0.112	0.309	0.895
Bait vs Control								
Z-test	-2.819	-3.584	-4.546	-0.699	1.393	1.372	-0.057	0.497
P value	0.005	<0.001	<0.001	0.484	0.163	0.17	0.955	0.619
Bait vs noChoice								
Z-test	-3.481	-4.221	-5.471	-3.642	2.308	-0.977	0	-1.227
P value	<0.001	<0.001	<0.001	<0.001	0.021	0.329	1	0.22
Bait composition	Ethyl acetate Methyl propionate β-Cyclocitral S-Linalool	Ethyl acetate Methyl propionate β-Cyclocitral S-Linalool	Ethyl acetate Methyl propionate β-Cyclocitral S-Linalool	Ethyl acetate Methyl propionate β-Cyclocitral S-Linalool	Ethyl acetate Methyl propionate β-Cyclocitral S-Linalool R-Linalool	S-Linalool	β-Cyclocitral	Ethyl acetate

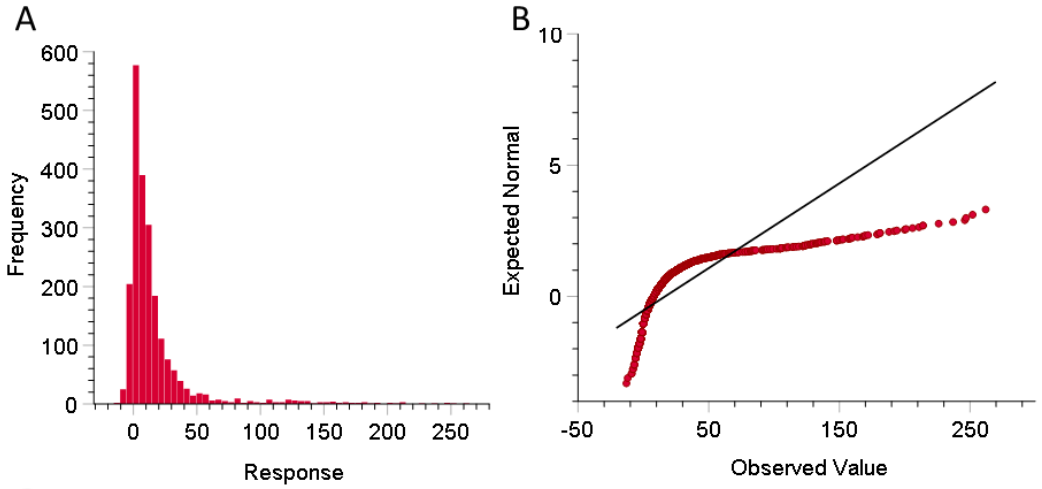
Table 10-11 The wind tunnel assay with chemicals: summary and statistics

Mean (+/- SEM) number of females which reached the target platforms (upwind attraction) and which chose the bait over the water (Choice) during 5h wind tunnel assay.

	Isoamyl acetate	**Blend 67:29:4**	**Hexane**
%Resp.	88 (±2)	66 (±7)	74 (±5)
Nrepl.	8	4	4
N*	16-36	20-23	17-27
Upwind attraction			
Nupwind	18 (±2)	11 (±2)	8 (±2)
Ndownwind	8 (±1)	11 (±2)	14 (±1)
t value[1]	-2.559	-1.137	
P value[1]	0.021	0.273	
PI-upwind	0.3 (±0.1)	0 (±0.2)	-0.3 (±0.1)
t-test	6.557	0.06263	2.82
P value	0.0003	0.9558	0.0667
upwind Distribution			
Nbait	9 (±1)	4 (±1)	5 (±1)
N water	8 (±1)	7 (±2)	3 (±0)
Chemical vs Hexane			
Zvalue[2]	-0.769	-2.005	
P value[2]	0.442	0.045	
Bait vs Control			
Nf, Nw[3]	82, 72	16, 27	20, 13
P value[3]	0.468	0.126	0.296
PI-Choice	0.1 (±0.1)	-0.2 (±0.2)	0.2 (±0.2)
t-test	1.069	0.6263	0.9015
P value	0.3206	0.5951	0.4338

"%Resp.", proportion of females collected upwind (Responders). *N females per N replication (Nrepl.). [1]Quasibinomial GLM on the distribution of females (upwind, downwind) in fruit experiments versus Control [Residual deviance: 54.641 on 17 degrees of freedom]; [2]Binomial GLM on the distribution of females on the two baits, experiments with chemicals versus Control (with Hexane) [Residual deviance: 21.81 on 17 Degrees of freedom]. [3]Binomial test on the number of females collected on the bait (Nf) and the water (Nw), within each experiment. Blend 67:29:4 is composed of 67% Isoamyl acetate, 29% 2-Methyl butyl acetate, 4% 4-hydroxy-4-methyl-2-pentanone.

10.7 APPENDIX 8. ORN RESPONSES TO FRUIT HEADSPACES IN D. MELANOGASTER



C

	Statistic	df	P value		Statistic	df	P value
All responses	0.544	2160	<0.001	ab4A	0.764	88	<0.001
Per stimuli				ab4B	0.976	88	0.103
Sp. act.	0.925	270	<0.001	ab5A	0.925	72	<0.001
Control	0.914	270	<0.001	ab5B	0.910	72	<0.001
Strawberry	0.658	270	<0.001	ab6A	0.962	48	0.117
Raspberry	0.689	270	<0.001	ab6B	0.867	48	<0.001
Blueberry	0.571	270	<0.001	ab7A	0.698	88	<0.001
Grape	0.580	270	<0.001	ab7B	0.732	88	<0.001
Orange	0.580	270	<0.001	ab8A	0.903	88	<0.001
Tomato	0.616	270	<0.001	ab8B	0.830	88	<0.001
Per ORN class				ab10A	0.956	40	0.125
ab1A	0.811	88	<0.001	ab10B	0.857	40	<0.001
ab1B	0.967	88	0.024	abYA	0.671	80	<0.001
ab1C	0.943	88	0.001	abYB	0.818	80	<0.001
ab1D	0.961	88	0.009	pb1A	0.673	88	<0.001
ab2A	0.740	80	<0.001	pb1B	0.964	88	0.016
ab2B	0.756	80	<0.001	pb2A	0.977	72	0.222
ab3A	0.826	80	<0.001	pb2B	0.916	72	<0.001
ab3B	0.915	80	<0.001	pb3A	0.961	80	0.017
				pb3B	0.966	80	0.032

Figure 10-4. Normality tests on ORN responses to fruit headspaces in D. melanogaster.

A) Distribution of data by frequency. B) QQ plot of measured data (dots) compared to an expected Gaussian distribution (line).C) Shapiro-Wilk normality test on the ORN responses to fruit headspaces. Variables are defined from the categorical variables of the dataset. All responses include all data, undivided. All belong to the same dataset. df: degree of freedom. P value <0.05 indicates that data do not follow a Gaussian distribution.

Table 10-12 Responses to fruit headspace by D. melanogaster classes of ORNs

Mean (± SEM) number of impulses per second during a 0.5 s stimulus with headspaces from harvested whole ripe strawberries, raspberries, blueberries, grapes, oranges and tomatoes, a control (ambient air). The spontaneous activity (baseline) was deducted from the responses to control and headspaces.

	Strawberry	Raspberry	Blueberry	Grape	Orange	Tomato	Control	Baseline	N
ab1A	125 (±11)	219 (±10)	89 (±15)	48 (±6)	39 (±3)	19 (±3)	21 (±2)	12 (±1)	11
ab1B	27 (±3)	28 (±3)	18 (±3)	18 (±4)	16 (±3)	10 (±3)	8 (±2)	7 (±2)	11
ab1C	16 (±2)	21 (±6)	18 (±3)	12 (±2)	14 (±3)	13 (±2)	10 (±2)	14 (±3)	11
ab1D	0 (±2)	-3 (±1)	2 (±2)	5 (±2)	7 (±2)	5 (±1)	7 (±2)	7 (±1)	11
ab2A	146 (±8)	126 (±15)	28 (±6)	19 (±2)	10 (±3)	7 (±3)	9 (±3)	7 (±2)	10
ab2B	9 (±3)	1 (±1)	0 (±1)	0 (±1)	6 (±4)	0 (±1)	4 (±1)	3 (±1)	10
ab3A	148 (±7)	51 (±7)	21 (±2)	35 (±7)	122 (±10)	10 (±4)	11 (±4)	7 (±2)	10
ab3B	11 (±3)	20 (±3)	6 (±1)	4 (±2)	5 (±2)	5 (±2)	8 (±1)	10 (±1)	10
ab4A	46 (±8)	20 (±5)	25 (±5)	120 (±16)	20 (±3)	61 (±7)	10 (±2)	13 (±2)	11
ab4B	5 (±2)	3 (±1)	2 (±1)	0 (±2)	4 (±2)	5 (±2)	5 (±2)	9 (±2)	11
ab5A	24 (±4)	9 (±2)	12 (±3)	13 (±3)	16 (±4)	13 (±3)	6 (±2)	7 (±3)	9
ab5B	15 (±3)	5 (±2)	3 (±2)	3 (±3)	7 (±4)	6 (±3)	5 (±3)	9 (±3)	9
ab6A	17 (±6)	7 (±4)	17 (±3)	25 (±3)	11 (±5)	8 (±5)	5 (±3)	9 (±3)	6
ab6B	15 (±5)	1 (±2)	4 (±1)	21 (±4)	3 (±2)	1 (±3)	6 (±3)	10 (±4)	6
ab7A	27 (±4)	20 (±5)	17 (±2)	25 (±3)	35 (±4)	104 (±13)	20 (±2)	13 (±2)	11
ab7B	16 (±7)	10 (±4)	6 (±3)	9 (±4)	13 (±7)	36 (±8)	5 (±2)	6 (±2)	11
ab8A	20 (±4)	14 (±5)	6 (±3)	6 (±3)	9 (±3)	4 (±3)	1 (±2)	10 (±2)	11
ab8B	13 (±4)	12 (±4)	1 (±1)	2 (±2)	4 (±2)	3 (±2)	1 (±1)	7 (±1)	11
ab9A	12 (±4)	3 (±2)	7 (±3)	7 (±4)	14 (±5)	6 (±3)	9 (±2)	17 (±5)	5
ab9B	8 (±3)	2 (±1)	10 (±2)	12 (±3)	10 (±1)	32 (±5)	8 (±3)	9 (±3)	5
ab10A	36 (±4)	1 (±1)	3 (±1)	5 (±2)	55 (±7)	4 (±1)	5 (±2)	3 (±1)	13
ab10B	4 (±1)	2 (±1)	2 (±1)	1 (±1)	5 (±1)	1 (±1)	0 (±0)	2 (±1)	13
pb1A	49 (±4)	73 (±15)	6 (±1)	8 (±1)	20 (±2)	15 (±2)	3 (±1)	11 (±1)	11
pb1B	6 (±2)	8 (±2)	5 (±2)	2 (±1)	3 (±1)	5 (±2)	3 (±1)	5 (±1)	11
pb2A	7 (±2)	5 (±3)	4 (±2)	4 (±2)	4 (±2)	8 (±1)	5 (±1)	7 (±2)	9
pb2B	6 (±3)	5 (±3)	4 (±2)	5 (±2)	5 (±2)	7 (±2)	2 (±1)	6 (±2)	9
pb3A	16 (±2)	7 (±2)	5 (±2)	6 (±1)	8 (±2)	8 (±2)	5 (±2)	8 (±2)	10
pb3B	13 (±3)	8 (±2)	5 (±2)	7 (±2)	8 (±3)	8 (±2)	7 (±2)	8 (±2)	10

Table 10-13. Statistical comparison of responses to fruit headspace and control by D. melanogaster classes of ORNs

Mann Whitney Exact test between responses to fruits and their respective controls for each ORN. Z is the statistical test, P the associated *P* value. Differences are significant when P<0.05. ***P<0.001, **P<0.01, *P<0.05.

	Strawberry	Raspberry	Blueberry	Grape	Orange	Tomato
ab1A	Z= -3.98 P= <0.001***	Z= -3.98 P= <0.001***	Z= 3.87 P= <0.001***	Z= -2.67 P= <0.01**	Z= -3.26 P= <0.001***	Z= 0.72 P= 0.48
ab1B	Z= -3.62 P= <0.001***	Z= -3.65 P= <0.001***	Z= 2.53 P= <0.01**	Z= -2.05 P= 0.04*	Z= -1.88 P= 0.06	Z= -0.6 P= 0.57
ab1C	Z= -1.98 P= 0.04*	Z= -1.68 P= 0.09	Z= 1.77 P= 0.07	Z= -0.9 P= 0.39	Z= -1.49 P= 0.14	Z= -1.19 P= 0.24
ab1D	Z= 2.53 P= <0.01**	Z= 3.88 P= <0.001***	Z= -1.95 P= 0.05	Z= 0.65 P= 0.52	Z= 0.19 P= 0.85	Z= 0.62 P= 0.54
abYA	Z= -3.57 P= <0.001***	Z= 0.99 P= 0.33	Z= -0.43 P= 0.69	Z= 0.38 P= 0.72	Z= -3.76 P= <0.001***	Z= -0.43 P= 0.69
abYB	Z= -2.87 P= <0.01**	Z= -1.17 P= 0.25	Z= 2.11 P= 0.03*	Z= -1.5 P= 0.18	Z= -2.55 P= <0.01**	Z= -1.58 P= 0.12
ab2A	Z= -3.79 P= <0.001***	Z= -3.79 P= <0.001***	Z= 2.3 P= 0.01*	Z= -2.13 P= 0.03*	Z= -0.27 P= 0.8	Z= 0.68 P= 0.51
ab2B	Z= -1.87 P= 0.06	Z= 1.71 P= 0.08	Z= -2.15 P= 0.03*	Z= 2.41 P= 0.01*	Z= 0.72 P= 0.48	Z= 2.29 P= 0.02*
ab4A	Z= -3.72 P= <0.001***	Z= -1.22 P= 0.23	Z= 2.73 P= <0.01**	Z= -3.98 P= <0.001***	Z= -2.31 P= 0.01*	Z= -3.98 P= <0.001***
ab4B	Z= 0.23 P= 0.83	Z= 1.08 P= 0.28	Z= -1.56 P= 0.12	Z= 1.81 P= 0.07	Z= 0.56 P= 0.59	Z= 0.03 P= 0.98
ab5A	Z= -3.28 P= <0.001***	Z= -0.94 P= 0.37	Z= 1.33 P= 0.19	Z= -1.82 P= 0.07	Z= -2.05 P= 0.04*	Z= -1.43 P= 0.16
ab5B	Z= -2.26 P= 0.02*	Z= -0.09 P= 0.94	Z= -0.95 P= 0.36	Z= 0.98 P= 0.34	Z= -0.09 P= 0.94	Z= 0.22 P= 0.84
ab6A	Z= -1.53 P= 0.14	Z= -0.65 P= 0.57	Z= 2.24 P= 0.02*	Z= -2.73 P= <0.01**	Z= -0.89 P= 0.41	Z= -0.17 P= 0.9
ab6B	Z= -1.21 P= 0.25	Z= 1.36 P= 0.19	Z= -0.17 P= 0.91	Z= -2.25 P= 0.02*	Z= 0.88 P= 0.41	Z= 0.88 P= 0.41
ab10A	Z= -0.95 P= 0.41	Z= 1.59 P= 0.13	Z= -0.22 P= 0.89	Z= 0.21 P= 0.89	Z= -1.16 P= 0.27	Z= 0.74 P= 0.54
ab10B	Z= -0.64 P= 0.57	Z= 1.37 P= 0.2	Z= 0.73 P= 0.54	Z= -1.68 P= 0.1	Z= -1.48 P= 0.15	Z= -2.52 P= 0.01*
ab3A	Z= -3.79 P= <0.001***	Z= -3.49 P= <0.001***	Z= 1.96 P= 0.04*	Z= -2.58 P= <0.01**	Z= -3.79 P= <0.001***	Z= 0.52 P= 0.61
ab3B	Z= -0.61 P= 0.56	Z= -2.85 P= <0.01**	Z= -1.07 P= 0.3	Z= 1.4 P= 0.16	Z= 1.1 P= 0.28	Z= 1.02 P= 0.32
ab7A	Z= -1.42 P= 0.16	Z= 0.55 P= 0.59	Z= -0.96 P= 0.35	Z= -1.16 P= 0.26	Z= -2.7 P= <0.01**	Z= -3.95 P= <0.001***
ab7B	Z= -1.42 P= 0.16	Z= -0.2 P= 0.85	Z= 0.13 P= 0.91	Z= -0.96 P= 0.35	Z= 0.13 P= 0.9	Z= -3.85 P= <0.001***
ab8A	Z= -3.63 P= <0.001***	Z= -2.28 P= 0.02*	Z= 1.19 P= 0.24	Z= -1.43 P= 0.16	Z= -2.12 P= 0.03*	Z= -1.23 P= 0.23
ab8B	Z= -2.91 P= <0.01**	Z= -2.38 P= 0.01*	Z= 0.43 P= 0.68	Z= -0.2 P= 0.86	Z= -0.77 P= 0.46	Z= 0.06 P= 0.96
pb1A	Z= -3.98 P= <0.001***	Z= -3.98 P= <0.001***	Z= 1.41 P= 0.16	Z= -2.08 P= 0.03*	Z= -3.95 P= <0.001***	Z= -3.43 P= <0.001***
pb1B	Z= -1.26 P= 0.21	Z= -2.29 P= 0.02*	Z= 0.03 P= 0.98	Z= 0.96 P= 0.34	Z= -0.04 P= 0.98	Z= -1.24 P= 0.22
pb3A	Z= -3.11 P= <0.001***	Z= -1.03 P= 0.32	Z= -0.16 P= 0.89	Z= -0.84 P= 0.42	Z= -0.92 P= 0.38	Z= -1.45 P= 0.15
pb3B	Z= -1.63 P= 0.1	Z= -0.19 P= 0.86	Z= -0.95 P= 0.36	Z= 0.18 P= 0.86	Z= -0.42 P= 0.69	Z= -0.57 P= 0.59
pb2A	Z= -0.45 P= 0.68	Z= 0.26 P= 0.81	Z= -0.72 P= 0.49	Z= 1.06 P= 0.3	Z= 0.97 P= 0.34	Z= -2.05 P= 0.04*
pb2B	Z= -1.13 P= 0.28	Z= -0.8 P= 0.44	Z= 0.44 P= 0.69	Z= -0.85 P= 0.42	Z= -1.3 P= 0.21	Z= -2.05 P= 0.04*

10.8 APPENDIX 9. COMPARISON OF ORN RESPONSES IN D. SUZUKII AND D. MELANOGASTER

Table 10-14 Comparative responses to ripe fruit headspaces in D. suzukii and D. melanogaster

Response: Mean (± SEM) impulses/s during a 0.5 s stimulus with headspace of ripe whole fruits. Comparison of responses between species using a Wilcoxon signed-ranked test followed by a Holm-Sidak correction for multiple comparisons. *P*<0.05, significant differences.

		D. suzukii		*D. melanogaster*			
ORN	**Headspace**	**Response**	**N**	**Response**	**N**	***Z stat***	***P value***
ab10A	Raspberry	35 (±5)	10	20 (±5)	5	-1.9	0.39
	Strawberry	39 (±5)	10	29 (±6)	5	-0.37	0.872
	Blueberry	36 (±4)	10	24 (±7)	5	-1.72	0.439
	Grape	30 (±4)	10	24 (±8)	5	-0.74	0.872
	Orange	33 (±3)	10	31 (±9)	5	-0.68	0.872
	Tomato	33 (±4)	10	23 (±7)	5	-1.35	0.648
	Control	34 (±3)	10	26 (±6)	5	-1.35	0.648
	Baseline	32 (±5)	10	17 (±5)	5	-1.79	0.439
ab10B	Raspberry	22 (±4)	10	11 (±2)	5	-1.6	0.405
	Strawberry	32 (±4)	10	17 (±3)	5	-2.52	0.063
	Blueberry	20 (±3)	10	19 (±3)	5	-0.12	0.929
	Grape	44 (±5)	10	21 (±4)	5	-2.95	0.013
	Orange	23 (±3)	10	19 (±3)	5	-0.86	0.663
	Tomato	50 (±4)	10	41 (±6)	5	-1.35	0.475
	Control	28 (±4)	10	16 (±5)	5	-1.84	0.32
	Baseline	20 (±3)	10	9 (±3)	5	-2.04	0.224
ab1A	Raspberry	136 (±10)	12	230 (±11)	11	3.82	<0.001
	Strawberry	123 (±14)	12	137 (±12)	11	0.99	0.809
	Blueberry	67 (±9)	12	101 (±15)	11	1.42	0.603
	Grape	60 (±3)	12	60 (±6)	11	0.25	0.938
	Orange	52 (±3)	12	51 (±3)	11	-0.34	0.938
	Tomato	43 (±4)	12	30 (±3)	11	-2.31	0.129
	Control	28 (±4)	12	33 (±2)	11	0.93	0.809
	Baseline	18 (±3)	12	12 (±1)	11	-1.98	0.255
ab1B	Raspberry	57 (±5)	12	35 (±3)	11	-2.8	0.026
	Strawberry	35 (±4)	12	34 (±3)	11	-0.37	0.728
	Blueberry	40 (±5)	12	25 (±3)	11	-2.09	0.167
	Grape	35 (±4)	12	24 (±5)	11	-1.79	0.27
	Orange	28 (±4)	12	23 (±4)	11	-0.77	0.706
	Tomato	24 (±3)	12	17 (±3)	11	-1.52	0.354
	Control	27 (±3)	12	15 (±3)	11	-2.2	0.152
	Baseline	20 (±3)	12	7 (±2)	11	-2.97	0.016
ab1C	Raspberry	47 (±4)	12	35 (±6)	11	-1.36	0.34
	Strawberry	47 (±5)	12	30 (±4)	11	-2.37	0.079
	Blueberry	43 (±2)	12	32 (±3)	11	-2.47	0.069
	Grape	44 (±3)	12	26 (±4)	11	-2.9	0.021
	Orange	40 (±5)	12	28 (±5)	11	-1.54	0.34
	Tomato	39 (±3)	12	26 (±3)	11	-2.62	0.049
	Control	28 (±4)	12	24 (±4)	11	-0.59	0.576
	Baseline	22 (±2)	12	14 (±3)	11	-2.07	0.144

ab1D	Raspberry	3 (±2)	12	3 (±1)	11	1.45	0.688
	Strawberry	5 (±1)	12	7 (±1)	11	1.09	0.872
	Blueberry	12 (±2)	12	9 (±2)	11	-0.99	0.872
	Grape	15 (±3)	12	12 (±2)	11	-0.74	0.872
	Orange	15 (±2)	12	14 (±2)	11	-0.43	0.872
	Tomato	10 (±2)	12	12 (±1)	11	1.92	0.372
	Control	11 (±2)	12	14 (±1)	11	1.05	0.872
	Baseline	10 (±2)	12	7 (±1)	11	-1.08	0.872
ab2A	Raspberry	111 (±8)	12	133 (±16)	10	0.89	0.915
	Strawberry	150 (±14)	12	153 (±8)	10	0.03	0.987
	Blueberry	35 (±8)	12	35 (±6)	10	0.3	0.975
	Grape	28 (±4)	12	25 (±3)	10	-0.4	0.975
	Orange	34 (±8)	12	17 (±3)	10	-1.32	0.731
	Tomato	25 (±3)	12	14 (±3)	10	-2.25	0.152
	Control	18 (±2)	12	16 (±4)	10	-0.83	0.915
	Baseline	16 (±2)	12	7 (±2)	10	-2.78	0.032
ab2B	Raspberry	11 (±3)	12	4 (±1)	10	-2.18	0.055
	Strawberry	20 (±5)	12	12 (±3)	10	-1.03	0.319
	Blueberry	34 (±6)	12	3 (±1)	10	-3.11	0.003
	Grape	34 (±3)	12	3 (±1)	10	-3.97	<0.001
	Orange	35 (±4)	12	9 (±4)	10	-3.41	<0.001
	Tomato	40 (±3)	12	3 (±1)	10	-3.99	<0.001
	Control	40 (±2)	12	6 (±1)	10	-3.97	<0.001
	Baseline	36 (±2)	12	3 (±1)	10	-3.98	<0.001
ab3A	Raspberry	72 (±10)	15	58 (±6)	10	-1.08	0.586
	Strawberry	143 (±6)	15	155 (±7)	10	0.89	0.586
	Blueberry	56 (±6)	15	28 (±3)	10	-3.02	0.008
	Grape	32 (±4)	15	42 (±7)	10	1.17	0.586
	Orange	67 (±8)	15	129 (±10)	10	3.39	0.002
	Tomato	51 (±3)	15	17 (±5)	10	-3.8	<0.001
	Control	34 (±4)	15	18 (±4)	10	-2.61	0.03
	Baseline	22 (±2)	15	7 (±2)	10	-3.34	0.002
ab3B	Raspberry	43 (±5)	15	30 (±3)	10	-1.72	0.308
	Strawberry	36 (±3)	15	21 (±3)	10	-3	0.014
	Blueberry	29 (±3)	15	16 (±2)	10	-2.69	0.039
	Grape	19 (±3)	15	14 (±2)	10	-0.82	0.607
	Orange	25 (±3)	15	15 (±2)	10	-2.58	0.049
	Tomato	20 (±3)	15	15 (±2)	10	-1.17	0.586
	Control	22 (±4)	15	18 (±2)	10	-0.92	0.607
	Baseline	18 (±2)	15	10 (±1)	10	-2.39	0.073
ab4A	Raspberry	38 (±6)	13	33 (±5)	11	-0.61	0.806
	Strawberry	35 (±2)	13	59 (±8)	11	2.71	0.042
	Blueberry	30 (±5)	13	38 (±4)	11	1.91	0.207
	Grape	88 (±9)	13	133 (±17)	11	2.29	0.12
	Orange	21 (±3)	13	32 (±3)	11	2.56	0.062
	Tomato	60 (±12)	13	74 (±6)	11	1.91	0.207
	Control	17 (±1)	13	23 (±2)	11	2.18	0.134
	Baseline	15 (±2)	13	13 (±2)	11	-0.56	0.806
ab4B	Raspberry	4 (±1)	13	13 (±3)	11	2.31	0.113
	Strawberry	7 (±2)	13	15 (±2)	11	2.57	0.06
	Blueberry	8 (±2)	13	11 (±3)	11	0.99	0.703
	Grape	5 (±2)	13	10 (±2)	11	1.68	0.337
	Orange	13 (±4)	13	14 (±3)	11	0.61	0.802

	Tomato	4 (±1)	13	15 (±3)	11	3.13	0.008
	Control	8 (±2)	13	15 (±3)	11	1.9	0.258
	Baseline	8 (±2)	13	9 (±2)	11	0.56	0.802
ab5A	Raspberry	13 (±4)	10	17 (±4)	9	0.66	0.978
	Strawberry	26 (±3)	10	32 (±4)	9	0.78	0.975
	Blueberry	12 (±4)	10	19 (±5)	9	0.62	0.978
	Grape	12 (±4)	10	20 (±5)	9	1.31	0.795
	Orange	20 (±6)	10	24 (±6)	9	0.45	0.978
	Tomato	10 (±4)	10	20 (±5)	9	1.57	0.656
	Control	11 (±3)	10	13 (±5)	9	0.29	0.978
	Baseline	9 (±4)	10	7 (±3)	9	0.21	0.978
ab5B	Raspberry	32 (±6)	10	14 (±4)	9	-2.04	0.189
	Strawberry	32 (±3)	10	24 (±4)	9	-1.72	0.189
	Blueberry	30 (±5)	10	12 (±5)	9	-2.42	0.094
	Grape	35 (±6)	10	12 (±5)	9	-2.62	0.054
	Orange	35 (±7)	10	16 (±6)	9	-2.04	0.189
	Tomato	34 (±7)	10	15 (±5)	9	-1.8	0.189
	Control	36 (±8)	10	15 (±5)	9	-2.05	0.189
	Baseline	33 (±7)	10	9 (±3)	9	-2.37	0.094
ab6A	Raspberry	23 (±3)	10	16 (±2)	6	-2.07	0.254
	Strawberry	32 (±2)	10	26 (±5)	6	-0.87	0.881
	Blueberry	29 (±4)	10	26 (±3)	6	-0.38	0.981
	Grape	31 (±3)	10	34 (±2)	6	0.22	0.981
	Orange	20 (±4)	10	20 (±4)	6	-0.11	0.981
	Tomato	25 (±4)	10	16 (±3)	6	-1.47	0.635
	Control	23 (±4)	10	13 (±2)	6	-1.96	0.312
	Baseline	13 (±3)	10	9 (±3)	6	-1.25	0.72
ab6B	Raspberry	28 (±5)	10	11 (±3)	6	-2.39	0.112
	Strawberry	31 (±3)	10	25 (±5)	6	-0.87	0.728
	Blueberry	27 (±3)	10	14 (±4)	6	-2.17	0.137
	Grape	27 (±4)	10	31 (±5)	6	0.76	0.728
	Orange	20 (±4)	10	13 (±3)	6	-0.98	0.728
	Tomato	24 (±4)	10	11 (±1)	6	-2.23	0.137
	Control	26 (±5)	10	16 (±5)	6	-1.14	0.724
	Baseline	27 (±5)	10	10 (±4)	6	-2.39	0.112
ab7A	Raspberry	46 (±7)	12	33 (±5)	11	-1.2	0.646
	Strawberry	48 (±5)	12	40 (±5)	11	-1.36	0.638
	Blueberry	32 (±4)	12	30 (±3)	11	0	1
	Grape	36 (±3)	12	38 (±4)	11	0.46	0.885
	Orange	35 (±3)	12	48 (±5)	11	2.46	0.094
	Tomato	92 (±9)	12	117 (±14)	11	1.23	0.646
	Control	21 (±3)	12	33 (±3)	11	2.44	0.094
	Baseline	18 (±2)	12	13 (±2)	11	-1.45	0.631
ab7B	Raspberry	17 (±3)	12	17 (±6)	11	-1.32	0.695
	Strawberry	30 (±6)	12	22 (±9)	11	-1.33	0.695
	Blueberry	14 (±2)	12	12 (±4)	11	-1.37	0.695
	Grape	13 (±2)	12	15 (±5)	11	-0.71	0.744
	Orange	14 (±2)	12	19 (±9)	11	-1.88	0.394
	Tomato	33 (±6)	12	42 (±10)	11	0.31	0.772
	Control	17 (±4)	12	11 (±4)	11	-1.05	0.695
	Baseline	14 (±4)	12	6 (±2)	11	-1.61	0.561
ab8A	Raspberry	18 (±3)	12	24 (±5)	11	0.52	0.978
	Strawberry	39 (±6)	12	29 (±5)	11	-1.2	0.747

	Blueberry	18 (±3)	12	16 (±3)	11	-0.43	0.978
	Grape	17 (±3)	12	16 (±3)	11	-0.03	0.988
	Orange	18 (±3)	12	18 (±4)	11	0.22	0.978
	Tomato	18 (±3)	12	14 (±4)	11	-1.48	0.664
	Control	16 (±3)	12	11 (±2)	11	-1.49	0.664
	Baseline	16 (±3)	12	10 (±2)	11	-1.55	0.664
ab8B	Raspberry	15 (±4)	12	19 (±4)	11	0.95	0.888
	Strawberry	32 (±5)	12	20 (±4)	11	-1.79	0.469
	Blueberry	14 (±4)	12	8 (±2)	11	-0.68	0.888
	Grape	15 (±5)	12	9 (±2)	11	-0.93	0.888
	Orange	10 (±2)	12	11 (±3)	11	0.59	0.888
	Tomato	12 (±3)	12	10 (±3)	11	-0.28	0.888
	Control	13 (±3)	12	8 (±2)	11	-1.36	0.703
	Baseline	12 (±3)	12	7 (±1)	11	-1.56	0.608
pb1A	Raspberry	52 (±4)	11	84 (±15)	11	2.15	0.146
	Strawberry	105 (±14)	11	59 (±4)	11	-3.43	0.001
	Blueberry	90 (±8)	11	16 (±1)	11	-3.99	<0.001
	Grape	22 (±3)	11	18 (±1)	11	-1.16	0.698
	Orange	27 (±2)	11	30 (±2)	11	0.93	0.698
	Tomato	21 (±3)	11	26 (±3)	11	0.49	0.698
	Control	17 (±2)	11	14 (±2)	11	-1.09	0.698
	Baseline	15 (±2)	11	11 (±1)	11	-2.23	0.142
pb1B	Raspberry	19 (±3)	11	13 (±2)	11	-1.65	0.279
	Strawberry	13 (±2)	11	11 (±1)	11	-0.73	0.58
	Blueberry	13 (±2)	11	10 (±2)	11	-0.96	0.58
	Grape	21 (±4)	11	7 (±1)	11	-2.42	0.083
	Orange	19 (±4)	11	9 (±1)	11	-2.01	0.19
	Tomato	23 (±5)	11	11 (±2)	11	-2.04	0.19
	Control	25 (±4)	11	8 (±1)	11	-3.29	0.003
	Baseline	23 (±3)	11	5 (±1)	11	-3.86	<0.001
pb2A	Raspberry	25 (±5)	12	12 (±2)	9	-2.75	0.01
	Strawberry	33 (±5)	12	14 (±2)	9	-3.38	0.001
	Blueberry	28 (±5)	12	11 (±2)	9	-3.15	0.003
	Grape	19 (±2)	12	10 (±2)	9	-2.82	0.01
	Orange	23 (±1)	12	11 (±2)	9	-3.5	<0.001
	Tomato	26 (±2)	12	15 (±1)	9	-3.28	0.002
	Control	21 (±2)	12	12 (±2)	9	-2.64	0.01
	Baseline	20 (±1)	12	7 (±2)	9	-3.54	<0.001
pb2B	Raspberry	31 (±4)	11	11 (±3)	9	-2.85	0.012
	Strawberry	30 (±4)	11	12 (±4)	9	-2.55	0.018
	Blueberry	30 (±4)	11	9 (±3)	9	-3.05	0.007
	Grape	29 (±4)	11	11 (±3)	9	-2.51	0.018
	Orange	31 (±4)	11	11 (±3)	9	-2.78	0.012
	Tomato	29 (±3)	11	13 (±3)	9	-2.93	0.01
	Control	32 (±4)	11	8 (±3)	9	-3.31	0.002
	Baseline	32 (±5)	11	6 (±2)	9	-3.58	<0.001
pb3A	Raspberry	13 (±2)	12	16 (±2)	10	1.06	0.887
	Strawberry	15 (±2)	12	25 (±2)	10	2.71	0.04
	Blueberry	14 (±2)	12	13 (±2)	10	-0.76	0.887
	Grape	14 (±2)	12	14 (±2)	10	0.1	0.941
	Orange	13 (±2)	12	16 (±3)	10	1.02	0.887
	Tomato	15 (±2)	12	16 (±3)	10	0.89	0.887
	Control	13 (±2)	12	14 (±3)	10	-0.33	0.941

	Baseline	13 (±2)	12	8 (±2)	10	-1.36	0.76
pb3B	Raspberry	14 (±2)	12	16 (±3)	10	0.37	0.93
	Strawberry	27 (±4)	12	21 (±3)	10	-0.96	0.824
	Blueberry	14 (±2)	12	13 (±3)	10	-0.2	0.93
	Grape	12 (±2)	12	15 (±3)	10	0.96	0.824
	Orange	10 (±2)	12	16 (±3)	10	1.75	0.498
	Tomato	13 (±2)	12	16 (±2)	10	1.33	0.781
	Control	11 (±2)	12	15 (±2)	10	1.23	0.781
	Baseline	12 (±2)	12	8 (±2)	10	-1.3	0.781

Table 10-15 Comparative responses to fruit chemicals in D. suzukii and D. melanogaster

Response: Mean (± SEM) impulses/s during a 0.5 s stimulus with 1% dose on filter paper of chemicals in paraffin oil (solvent). Comparison of responses between species using a Wilcoxon signed-ranked test followed by a Holm-Sidak correction for multiple comparisons. P<0.05, significant differences. Enantiomer (1R,4aS,7S,7aR)-nepetalactol.

		D. suzukii		***D. melanogaster***			
ORN	**Headspace**	**Response**	**N**	**Response**	**N**	***Z stat***	***P value***
ab10A	Spontaneous activity	29 (±3)	13	12 (±2)	6	-3.27	0.009
	Nepetalactol	99 (±15)	4	11 (± 7)	2	-1.85	0.924
	Sulcatone	96 (±9)	9	59 (±13)	3	-1.85	0.803
	(RS)- 1-Octen-3-ol	184 (±17)	9	106 (±29)	3	-1.85	0.803
	Pentyl acetate	166 (±15)	9	63 (±13)	3	-2.5	0.219
	Paraffin oil	4 (±4)	13	-3 (±2)	6	-1.11	0.989
	Methyl salicylate	97 (±18)	10	135 (±39)	3	1.01	0.994
	Isoamyl acetate	112 (±13)	9	56 (±10)	3	-1.94	0.782
	Geranyl acetate	12 (±6)	6	19 (±8)	3	0.78	0.999
	Ethyl lactate	65 (±25)	8	4 (±1)	4	-1.87	0.803
	Ethyl butanoate	76 (±11)	9	32 (±4)	3	-1.95	0.782
	Ethyl benzoate	98 (±19)	10	41 (±20)	4	-1.84	0.803
	Ethyl acetate	21 (±10)	9	-4 (±3)	3	-1.3	0.987
	Diethyl succinate	184 (± 9)	4				
	CO_2 (Breath)	23 (±9)	9	-2 (±3)	3	-1.77	0.851
	Beta-cyclocitral	12 (±6)	9	9 (±4)	3	-0.37	1
	Benzaldehyde	138 (±17)	5	95 (±26)	4	-1.11	0.99
	Alpha-Terpineol	15 (±7)	6	45 (±19)	4	1.18	0.989
	Acetophenone	86 (± 21)	4				
	2-Phenylethanol	197 (±13)	6	201 (±18)	4	0	1
	2-Heptanone	138 (±12)	9	85 (±27)	3	-1.57	0.931
	2,3-Butanedione	12 (±7)	9	-1 (±5)	3	-1.21	0.989
	1-Hexanol	161 (±15)	9	154 (±36)	3	-0.09	1
	(RS)-Linalool	129 (±21)	6	106 (±30)	3	-0.26	1
	(E)-2-Hexenal	98 (±13)	9	34 (±6)	3	-2.5	0.219
ab10B	Spontaneous activity	23 (±3)	13	5 (±2)	6	-3.12	0.015
	Nepetalactol	15 (±7)	4	8 (± 0)	2	-0.94	1

Sulcatone	36 (±10)	9	35 (±4)	3	-0.37	1
(RS)- 1-Octen-3-ol	32 (±8)	9	-5 (±4)	3	-2.5	0.219
Pentyl acetate	39 (±17)	9	-1 (±5)	3	-1.94	0.795
Paraffin oil	0 (±4)	13	3 (±2)	6	0.84	1
Methyl salicylate	5 (±5)	10	-2 (±4)	3	-0.85	1
Isoamyl acetate	-5 (±5)	9	-5 (±3)	3	0.19	1
Geranyl acetate	-16 (±5)	6	-1 (±4)	3	1.57	0.992
Ethyl lactate	3 (±8)	8	4 (±3)	4	-0.17	1
Ethyl butanoate	-6 (±6)	9	-6 (±4)	3	-0.28	1
Ethyl benzoate	2 (±8)	10	-1 (±1)	4	0.43	1
Ethyl acetate	-5 (±5)	9	0 (±4)	3	0.74	1
Diethyl succinate	10 (± 19)	4				
CO_2 (Breath)	-1 (±4)	9	-1 (±1)	3	0.28	1
Beta-cyclocitral	1 (±6)	9	2 (±5)	3	0.28	1
Benzaldehyde	48 (±5)	5	46 (±18)	4	0	1
Alpha-Terpineol	13 (±6)	6	-1 (±3)	4	-1.71	0.93
Acetophenone	174 (± 17)	4				
2-Phenylethanol	55 (±18)	6	24 (±6)	4	-1.71	0.918
2-Heptanone	50 (±14)	9	60 (±15)	3	-0.09	1
2,3-Butanedione	-5 (±3)	9	-1 (±3)	3	0.75	1
1-Hexanol	59 (±17)	9	70 (±15)	3	0.46	1
(RS)-Linalool	7 (±8)	6	-2 (±6)	3	-0.52	1
(E)-2-Hexenal	19 (±10)	9	26 (±1)	3	0.28	1
ab1A Spontaneous activity	21 (±3)	7	12 (±3)	6	-2	0.699
Nepetalactol	30 (±0)	1	102	1	1.41	0.998
Sulcatone	29 (±10)	9	42 (±15)	3	0.83	1
(RS)- 1-Octen-3-ol	49 (±4)	3	26 (±16)	3	-1.2	0.996
Pentyl acetate	79 (±11)	3	79 (±4)	3	1.02	0.998
Paraffin oil	21 (±6)	7	33 (±9)	6	1.29	0.988
Methyl salicylate	20 (±9)	3	55 (±26)	3	1.48	0.971
Isoamyl acetate	99 (±8)	2	113 (±23)	3	0.35	1
Geranyl acetate	74 (±8)	3	85 (±9)	3	1.22	0.996
Ethyl lactate	155 (±15)	2	87 (±63)	2	-1.15	0.999
Ethyl butanoate	187 (±13)	3	193 (±33)	3	0.28	1
Ethyl benzoate	29 (±1)	2	39 (±18)	3	0.66	1
Ethyl acetate	179 (±13)	3	202 (±26)	3	0.83	1
CO_2 (Breath)	-6 (±3)	4	3 (±6)	3	1.43	0.985
Beta-cyclocitral	74 (±14)	3	98 (±61)	3	-0.28	1
Benzaldehyde	17 (±6)	3	35 (±13)	3	1.64	0.966
Alpha-Terpineol	6 (±6)	3	35 (±3)	3	2.25	0.639
2-Phenylethanol	11 (±4)	3	30 (±6)	3	1.94	0.831
2-Heptanone	115 (±15)	3	131 (±18)	3	0.74	1
2,3-Butanedione	44 (±11)	3	27 (±11)	3	-0.83	1
1-Hexanol	102 (±8)	3	126 (±5)	3	1.94	0.754
(RS)-Linalool	-6 (±3)	3	11 (±1)	3	2.25	0.639
(E)-2-Hexenal	68 (±7)	3	111 (±30)	3	1.67	0.93

ab1B Spontaneous activity	12 (±2)	7	13 (±3)	6	0.63	1
Nepetalactol	33 (±29)	1	52	1	0	1
Sulcatone	26 (±9)	9	-3 (±7)	3	-1.57	0.986
(RS)- 1-Octen-3-ol	7 (±4)	3	25 (±10)	3	1.48	0.989
Pentyl acetate	6 (±6)	3	12 (±8)	3	0.19	1
Paraffin oil	39 (±9)	7	18 (±6)	6	-1.07	1
Methyl salicylate	30 (±17)	3	18 (±10)	3	0.09	1
Isoamyl acetate	12 (±8)	2	14 (±6)	3	0.34	1
Geranyl acetate	23 (±5)	3	17 (±13)	3	-0.41	1
Ethyl lactate	26 (±11)	2	40 (±32)	2	0.58	1
Ethyl butanoate	26 (±5)	3	22 (±4)	3	-0.47	1
Ethyl benzoate	9 (±4)	2	11 (±7)	3	0	1
Ethyl acetate	28 (±7)	3	17 (±7)	3	-0.65	1
CO_2 (Breath)	-1 (±4)	4	4 (±3)	3	1.58	0.98
Beta-cyclocitral	11 (±4)	3	12 (±15)	3	0.37	1
Benzaldehyde	20 (±14)	3	21 (±6)	3	0.45	1
Alpha-Terpineol	31 (±15)	3	16 (±10)	3	-0.15	1
2-Phenylethanol	24 (±13)	3	13 (±5)	3	0	1
2-Heptanone	12 (±4)	3	11 (±14)	3	0.37	1
2,3-Butanedione	106 (±7)	3	91 (±23)	3	-0.46	1
1-Hexanol	23 (±7)	3	10 (±3)	3	-1.11	1
(RS)-Linalool	19 (±15)	3	9 (±9)	3	-0.15	1
(E)-2-Hexenal	21 (±7)	3	29 (±7)	3	0.56	1
ab1C Spontaneous activity	18 (±2)	8	9 (±3)	6	-2.27	0.459
Nepetalactol	18 (±16)	1	40	1	1.22	1
Sulcatone	-8 (±4)	9	3 (±5)	3	1.48	0.986
(RS)- 1-Octen-3-ol	-5 (±4)	3	13 (±11)	3	1.86	0.86
Pentyl acetate	7 (±7)	3	11 (±4)	3	0.75	1
Paraffin oil	19 (±6)	8	14 (±3)	6	-0.55	1
Methyl salicylate	6 (±5)	3	10 (±6)	3	1.02	1
Isoamyl acetate	7 (±11)	2	25 (±1)	3	1.27	0.997
Geranyl acetate	13 (±7)	3	13 (±5)	3	0	1
Ethyl lactate	12 (±10)	2	5 (±5)	2	-0.58	1
Ethyl butanoate	7 (±7)	3	-7 (±7)	3	-0.84	1
Ethyl benzoate	10 (±5)	2	18 (±5)	3	1.12	1
Ethyl acetate	-5 (±3)	3	5 (±4)	3	1.58	0.98
CO_2 (Breath)	174 (±16)	4	135 (±15)	3	-1.55	0.986
Beta-cyclocitral	-1 (±4)	3	9 (±2)	3	1.21	0.998
Benzaldehyde	16 (±7)	4	19 (±3)	3	-0.15	1
Alpha-Terpineol	14 (±8)	4	19 (±6)	3	0.3	1
2-Phenylethanol	6 (±9)	4	15 (±5)	3	0.75	1
2-Heptanone	6 (±8)	3	3 (±5)	3	-0.46	1
2,3-Butanedione	20 (±10)	3	28 (±12)	3	0.56	1
1-Hexanol	10 (±7)	3	9 (±4)	3	-0.28	1
(RS)-Linalool	0 (±3)	4	1 (±4)	3	0.3	1
(E)-2-Hexenal	7 (±5)	3	27 (±9)	3	2.05	0.632

ab1D Spontaneous activity	11 (±2)	8	11 (±2)	6	0	1
Nepetalactol	98 (±84)	1	4	1	-1.22	1
Sulcatone	-1 (±4)	9	-7 (±1)	3	-0.47	1
(RS)- 1-Octen-3-ol	-7 (±4)	3	-10 (±2)	3	-0.28	1
Pentyl acetate	-7 (±3)	3	-3 (±2)	3	0.84	1
Paraffin oil	0 (±3)	8	-1 (±2)	6	0.15	1
Methyl salicylate	170 (±15)	3	210 (±16)	3	1.2	1
Isoamyl acetate	-7 (±3)	2	-6 (±3)	3	0	1
Geranyl acetate	-7 (±3)	3	-7 (±1)	3	-0.1	1
Ethyl lactate	-17 (±9)	2	-2 (±18)	2	0.59	1
Ethyl butanoate	-10 (±3)	3	-12 (±4)	3	0	1
Ethyl benzoate	223 (±6)	2	181 (±10)	3	-1.96	0.942
Ethyl acetate	-13 (±4)	3	-1 (±3)	3	1.78	0.94
CO_2 (Breath)	-9 (±6)	4	-3 (±2)	3	0.26	1
Beta-cyclocitral	-9 (±3)	3	-7 (±1)	3	-0.09	1
Benzaldehyde	202 (±14)	4	87 (±53)	3	-1.64	0.982
Alpha-Terpineol	-2 (±2)	4	-1 (±3)	3	0	1
2-Phenylethanol	1 (±3)	4	6 (±4)	3	1.27	0.999
2-Heptanone	-7 (±4)	3	-7 (±4)	3	-0.28	1
2,3-Butanedione	-6 (±3)	3	-12 (±4)	3	-0.75	1
1-Hexanol	-4 (±2)	3	0 (±3)	3	1.12	1
(RS)-Linalool	1 (±3)	4	-5 (±2)	3	-1.67	0.993
(E)-2-Hexenal	-4 (±4)	3	-7 (±1)	3	-0.47	1
ab2A Spontaneous activity	12 (±1)	37	10 (±2)	8	-0.47	1
Nepetalactol	27 (±5)	1	76 (± 68)	2	0	1
Sulcatone	-3 (±3)	34	-11 (±6)	3	-1.09	0.997
(RS)- 1-Octen-3-ol	-6 (±2)	29	-8 (±6)	3	-0.22	1
Pentyl acetate	21 (±4)	29	37 (±7)	3	1.11	0.997
Paraffin oil	8 (±2)	35	0 (±3)	8	-1.86	0.777
Methyl salicylate	8 (±3)	29	-9 (±5)	3	-1.87	0.777
Isoamyl acetate	18 (±6)	17	53 (±7)	3	1.93	0.753
Geranyl acetate	41 (±5)	29	19 (±7)	2	-1.07	0.998
Ethyl lactate	136 (±29)	4	13 (±12)	4	-2.32	0.543
Ethyl butanoate	71 (±4)	29	32 (±1)	3	-2.65	0.053
Ethyl benzoate	-3 (±3)	0	-2 (±1)	3	0	1
Ethyl acetate	177 (±9)	29	177 (±9)	3	-0.33	1
Diethyl succinate			-2	1		
CO_2 (Breath)	7 (±2)	3	-2 (±8)	3	-1.41	0.978
Beta-cyclocitral	2 (±2)	29	5 (±4)	3	0.81	1
Benzaldehyde	10 (±5)	4	-10 (±2)	3	-2.12	0.756
Alpha-Terpineol	2 (±3)	5	-7 (±2)	3	-2	0.789
Acetophenone		0	-6	1		
2-Phenylethanol	2 (±4)	5	-8 (±1)	3	-2.25	0.612
2-Heptanone	18 (±4)	29	17 (±11)	3	0.11	1
2,3-Butanedione	117 (±11)	29	57 (±6)	3	-1.61	0.902
1-Hexanol	13 (±3)	29	6 (±3)	3	-0.28	1

(RS)-Linalool	-1 (±6)	4	-6 (±2)	3	-0.36	1
(E)-2-Hexenal	18 (±3)	29	-1 (±5)	3	-1.73	0.839
ab2B Spontaneous activity	37 (±2)	37	4 (±2)	8	-4.45	<0.001
Nepetalactol	60 (±24)	1	1 (± 1)	2	-1.55	0.995
Sulcatone	124 (±10)	34	27 (±3)	3	-2.48	0.132
(RS)- 1-Octen-3-ol	87 (±5)	29	25 (±3)	3	-2.84	0.007
Pentyl acetate	113 (±7)	29	13 (±6)	3	-2.73	0.025
Paraffin oil	16 (±5)	35	2 (±2)	8	-1.11	0.989
Methyl salicylate	2 (±3)	29	1 (±1)	3	0.06	1
Isoamyl acetate	94 (±9)	17	29 (±7)	3	-2.23	0.362
Geranyl acetate	25 (±6)	29	-1 (±1)	2	-1.62	0.887
Ethyl lactate	-35 (±6)	4	81 (±33)	4	2.31	0.423
Ethyl butanoate	-8 (±6)	29	85 (±20)	3	2.62	0.055
Ethyl benzoate	20 (±12)	0	29 (±16)	3	0	1
Ethyl acetate	-33 (±4)	29	13 (±9)	3	2.65	0.046
Diethyl succinate			2	1		
CO_2 (Breath)	6 (±6)	3	1 (±2)	3	0	1
Beta-cyclocitral	9 (±4)	29	-1 (±2)	3	-1.23	0.983
Benzaldehyde	11 (±9)	4	10 (±5)	3	0.18	1
Alpha-Terpineol	2 (±10)	5	5 (±3)	3	-0.15	1
Acetophenone		0	0	1		
2-Phenylethanol	2 (±10)	5	5 (±2)	3	-0.3	1
2-Heptanone	107 (±7)	29	5 (±5)	3	-2.84	0.007
2,3-Butanedione	-12 (±6)	29	5 (±4)	3	1.28	0.98
1-Hexanol	46 (±4)	29	55 (±6)	3	0.92	0.997
(RS)-Linalool	58 (±20)	4	5 (±5)	3	-1.78	0.887
(E)-2-Hexenal	44 (±5)	29	4 (±1)	3	-2.67	0.039
ab3A Spontaneous activity	18 (±3)	14	7 (±3)	8	-2.68	0.139
Nepetalactol	139 (±1)	1	124 (± 28)	2	0	1
Sulcatone	57 (±9)	18	30 (±11)	5	-1.49	0.949
(RS)- 1-Octen-3-ol	74 (±11)	10	110 (±25)	5	0.9	0.999
Pentyl acetate	157 (±9)	10	178 (±27)	5	0.82	0.999
Paraffin oil	16 (±4)	14	7 (±3)	8	-1.24	0.982
Methyl salicylate	9 (±3)	10	3 (±5)	5	-0.94	0.999
Isoamyl acetate	119 (±10)	9	176 (±25)	5	2.31	0.362
Geranyl acetate	22 (±5)	10	20 (±12)	4	-0.09	1
Ethyl lactate	93 (±15)	3	121 (±45)	3	0.56	1
Ethyl butanoate	164 (±14)	10	165 (±24)	5	-0.26	1
Ethyl benzoate	50 (±14)	3	20 (±10)	5	-1.47	0.953
Ethyl acetate	125 (±16)	10	32 (±8)	5	-2.27	0.383
CO_2 (Breath)	3 (±3)	3	1 (±2)	5	-0.81	0.999
Beta-cyclocitral	123 (±21)	10	8 (±6)	5	-2.69	0.124
Benzaldehyde	43 (±5)	3	6 (±6)	3	-1.96	0.902
Alpha-Terpineol	26 (±6)	3	4 (±2)	3	-1.99	0.902
2-Phenylethanol	27 (±2)	3	4 (±0)	3	-2.09	0.902
2-Heptanone	101 (±13)	10	69 (±11)	5	-1.23	0.982

2,3-Butanedione	91 (±10)	10	12 (±7)	5	-2.98	0.029
1-Hexanol	86 (±11)	10	72 (±14)	5	-0.6	1
(RS)-Linalool	27 (±10)	3	3 (±4)	3	-1.53	0.977
(E)-2-Hexenal	72 (±8)	10	45 (±9)	5	-1.83	0.81
ab3B Spontaneous activity	19 (±2)	14	12 (±3)	8	-1.72	0.866
Nepetalactol	57 (±7)	1	32 (± 6)	2	-1.55	0.996
Sulcatone	123 (±7)	18	163 (±33)	5	1.19	0.994
(RS)- 1-Octen-3-ol	111 (±13)	10	68 (±16)	5	-1.98	0.704
Pentyl acetate	116 (±13)	10	99 (±35)	5	-0.22	1
Paraffin oil	9 (±4)	14	32 (±8)	8	1.92	0.743
Methyl salicylate	-1 (±4)	10	19 (±7)	5	2.06	0.64
Isoamyl acetate	114 (±12)	9	56 (±26)	5	-1.9	0.768
Geranyl acetate	5 (±4)	10	31 (±13)	4	2.13	0.568
Ethyl lactate	31 (±16)	3	19 (±9)	3	0	1
Ethyl butanoate	32 (±18)	10	24 (±9)	5	1.04	0.996
Ethyl benzoate	11 (±7)	3	21 (±8)	5	1.27	0.992
Ethyl acetate	-1 (±5)	10	25 (±8)	5	2.43	0.297
CO_2 (Breath)	6 (±4)	3	-2 (±3)	5	-1.08	0.996
Beta-cyclocitral	18 (±6)	10	34 (±13)	5	1.12	0.995
Benzaldehyde	17 (±23)	3	13 (±8)	3	0.22	1
Alpha-Terpineol	17 (±3)	3	28 (±12)	3	0.66	1
2-Phenylethanol	11 (±3)	3	22 (±10)	3	0.65	1
2-Heptanone	159 (±9)	10	167 (±29)	5	0.19	1
2,3-Butanedione	41 (±7)	10	62 (±14)	5	1.23	0.994
1-Hexanol	123 (±16)	10	62 (±31)	5	-1.72	0.866
(RS)-Linalool	21 (±8)	3	29 (±12)	3	0.65	1
(E)-2-Hexenal	46 (±7)	10	30 (±10)	5	-1.23	0.994
ab4A Spontaneous activity	18 (±2)	17	12 (±2)	10	-1.47	0.981
Nepetalactol	39 (±3)	2	40 (± 8)	4	-0.18	1
Sulcatone	27 (±10)	15	13 (±5)	3	-0.6	1
(RS)- 1-Octen-3-ol	36 (±6)	13	13 (±8)	3	-1.66	0.956
Pentyl acetate	38 (±6)	13	60 (±31)	3	0.71	1
Paraffin oil	34 (±6)	17	23 (±7)	10	-1.23	0.997
Methyl salicylate	29 (±4)	13	18 (±8)	3	-0.89	1
Isoamyl acetate	17 (±5)	11	17 (±1)	3	0.61	1
Geranyl acetate	42 (±7)	13	21 (±5)	2	-1.5	0.991
Ethyl lactate	41 (±8)	7	66 (±37)	5	-0.22	1
Ethyl butanoate	30 (±6)	13	20 (±9)	3	-1.13	0.999
Ethyl benzoate	47 (±18)	7	9 (±7)	3	-1.67	0.958
Ethyl acetate	26 (±6)	13	32 (±13)	3	0.42	1
Diethyl succinate			1 (± 3)	2		
CO_2 (Breath)	11 (±5)	7	15 (±12)	3	0	1
Beta-cyclocitral	28 (±7)	13	11 (±4)	3	-1.01	1
Benzaldehyde	171 (±7)	2	161 (±6)	3	-1.09	1
Alpha-Terpineol	47 (±20)	2	20 (±10)	3	-1.09	1
Acetophenone		0	26 (± 4)	2		

2-Phenylethanol	51 (±16)	2	23 (±6)	3	-1.33	0.999
2-Heptanone	32 (±8)	13	17 (±9)	3	-0.77	1
2,3-Butanedione	43 (±5)	13	15 (±4)	3	-2.2	0.483
1-Hexanol	68 (±7)	13	69 (±34)	3	-0.59	1
(RS)-Linalool	43 (±8)	2	18 (±7)	3	-1.77	0.994
(E)-2-Hexenal	180 (±11)	13	192 (±7)	3	0.53	1
ab4B Spontaneous activity	9 (±1)	17	8 (±2)	10	-1.2	0.988
Nepetalactol	52 (±22)	2	14 (± 4)	4	-1.25	0.994
Sulcatone	16 (±3)	15	-5 (±6)	3	-2.31	0.351
(RS)- 1-Octen-3-ol	29 (±2)	13	1 (±4)	3	-2.56	0.098
Pentyl acetate	13 (±4)	13	13 (±8)	3	0.06	1
Paraffin oil	-3 (±2)	17	1 (±3)	10	1.51	0.939
Methyl salicylate	31 (±4)	13	0 (±6)	3	-2.37	0.259
Isoamyl acetate	21 (±5)	11	5 (±13)	3	-0.88	0.998
Geranyl acetate	5 (±2)	13	-9 (±9)	2	-1.51	0.943
Ethyl lactate	25 (±3)	7	51 (±40)	5	-0.95	0.998
Ethyl butanoate	26 (±3)	13	11 (±16)	3	-0.95	0.998
Ethyl benzoate	29 (±4)	7	-8 (±6)	3	-2.47	0.272
Ethyl acetate	7 (±3)	13	2 (±11)	3	0.06	1
Diethyl succinate			-2 (± 0)	2		
CO_2 (Breath)	-1 (±4)	7	-5 (±10)	3	-0.21	1
Beta-cyclocitral	40 (±7)	13	13 (±9)	3	-1.78	0.859
Benzaldehyde	9 (±3)	2	9 (±2)	3	0.22	1
Alpha-Terpineol	13 (±1)	2	-3 (±3)	3	-1.99	0.902
Acetophenone		0	6 (± 8)	2		
2-Phenylethanol	25 (±5)	2	2 (±1)	3	-1.96	0.902
2-Heptanone	20 (±3)	13	4 (±9)	3	-1.5	0.943
2,3-Butanedione	9 (±4)	13	1 (±8)	3	-0.77	0.999
1-Hexanol	34 (±5)	13	8 (±11)	3	-1.79	0.859
(RS)-Linalool	17 (±2)	2	-1 (±4)	3	-1.96	0.902
(E)-2-Hexenal	13 (±6)	13	3 (±5)	3	-0.42	1
ab5A Spontaneous activity	9 (±3)	18	4 (±3)	5	-0.66	1
Nepetalactol	10 (±5)	4	8 (± 2)	2	-0.46	1
Sulcatone	10 (±4)	13	13 (±6)	3	0.2	1
(RS)- 1-Octen-3-ol	31 (±6)	11	16 (±10)	3	-1.15	0.998
Pentyl acetate	23 (±6)	11	8 (±11)	3	-1.42	0.985
Paraffin oil	6 (±2)	18	7 (±2)	5	0.75	1
Methyl salicylate	4 (±3)	11	10 (±11)	3	0.2	1
Isoamyl acetate	110 (±14)	11	54 (±6)	3	-1.55	0.965
Geranyl acetate	134 (±16)	11	128 (±31)	3	-0.13	1
Ethyl lactate	8 (±5)	8	-1 (±4)	3	-1.36	0.988
Ethyl butanoate	36 (±5)	11	19 (±8)	3	-1.55	0.965
Ethyl benzoate	3 (±4)	8	12 (±8)	3	1.03	0.999
Ethyl acetate	32 (±6)	11	10 (±6)	3	-1.68	0.92
Diethyl succinate			14	1		
CO_2 (Breath)	-5 (±3)	8	11 (±6)	3	2.23	0.548

Beta-cyclocitral	2 (±3)	11	10 (±9)	3	0.54	1
Benzaldehyde	5 (±4)	3	-4 (±6)	2	-0.89	1
Alpha-Terpineol	8 (±8)	3	7 (±1)	2	0.59	1
Acetophenone		0	10	1		
2-Phenylethanol	12 (±8)	3	9 (±7)	2	-0.3	1
2-Heptanone	20 (±4)	11	2 (±5)	3	-1.95	0.773
2,3-Butanedione	10 (±4)	11	25 (±3)	3	1.95	0.762
1-Hexanol	31 (±6)	11	10 (±5)	3	-1.75	0.894
(RS)-Linalool	-1 (±1)	3	0 (±0)	2	0.82	1
(E)-2-Hexenal	12 (±3)	11	7 (±6)	3	-1.15	0.998
ab5B Spontaneous activity	36 (±4)	18	10 (±5)	5	-2.72	0.106
Nepetalactol	-5 (±8)	4	2 (± 2)	2	0.94	1
Sulcatone	5 (±11)	13	0 (±13)	3	0.13	1
(RS)- 1-Octen-3-ol	19 (±13)	11	11 (±7)	3	0.41	1
Pentyl acetate	159 (±12)	11	191 (±13)	3	1.01	0.999
Paraffin oil	-3 (±3)	18	1 (±2)	5	0.27	1
Methyl salicylate	-6 (±2)	11	1 (±4)	3	1.28	0.997
Isoamyl acetate	-2 (±10)	11	1 (±10)	3	0.61	1
Geranyl acetate	-26 (±6)	11	2 (±18)	3	1.28	0.997
Ethyl lactate	-9 (±5)	8	39 (±45)	3	0.68	1
Ethyl butanoate	2 (±11)	11	5 (±2)	3	1.55	0.975
Ethyl benzoate	-6 (±7)	8	-2 (±5)	3	0.25	1
Ethyl acetate	-8 (±5)	11	1 (±1)	3	1.28	0.997
Diethyl succinate			2	1		
CO_2 (Breath)	-1 (±5)	8	-3 (±1)	3	-1.03	0.999
Beta-cyclocitral	-3 (±3)	11	-5 (±11)	3	-0.14	1
Benzaldehyde	-3 (±9)	3	-3 (±3)	2	0.58	1
Alpha-Terpineol	12 (±2)	3	5 (±1)	2	-1.78	0.942
Acetophenone		0	2	1		
2-Phenylethanol	4 (±7)	3	2 (±6)	2	0	1
2-Heptanone	128 (±11)	11	95 (±28)	3	-1.28	0.997
2,3-Butanedione	-7 (±4)	11	3 (±1)	3	1.35	0.995
1-Hexanol	8 (±10)	11	3 (±3)	3	-0.34	1
(RS)-Linalool	19 (±5)	3	-1 (±5)	2	-1.73	0.995
(E)-2-Hexenal	-6 (±5)	11	1 (±4)	3	1.69	0.942
ab6A Spontaneous activity	12 (±2)	14	12 (±2)	8	0.65	0.998
Nepetalactol	152 (±11)	3	119 (± 55)	3	-0.75	0.998
Sulcatone	78 (±17)	10	23 (±14)	3	-1.69	0.831
(RS)- 1-Octen-3-ol	185 (±9)	10	176 (±32)	3	0	1
Pentyl acetate	130 (±17)	10	76 (±31)	3	-1.27	0.941
Paraffin oil	30 (±6)	14	4 (±4)	8	-2.75	0.107
Methyl salicylate	25 (±4)	10	-5 (±2)	3	-2.55	0.09
Isoamyl acetate	77 (±23)	8	0 (±6)	3	-2.35	0.32
Geranyl acetate	22 (±4)	10	3 (±6)	3	-2.03	0.641
Ethyl lactate	53 (±8)	8	-9 (±4)	5	-2.93	0.043
Ethyl butanoate	22 (±6)	10	-3 (±7)	3	-2.4	0.266

Ethyl benzoate	24 (±7)	8	-6 (±1)	3	-1.95	0.675
Ethyl acetate	27 (±5)	10	-7 (±1)	3	-2.55	0.161
Diethyl succinate			36	1		
CO_2 (Breath)	29 (±6)	8	-4 (±3)	3	-2.46	0.245
Beta-cyclocitral	27 (±5)	10	-2 (±3)	3	-2.37	0.224
Benzaldehyde	19 (±11)	1	-10 (±5)	4	-1.85	0.844
Alpha-Terpineol	29 (±3)	1	6 (±6)	4	-1.88	0.691
Acetophenone		0	24	1		
2-Phenylethanol	19 (±11)	1	-10 (±2)	4	-1.88	0.844
2-Heptanone	109 (±22)	10	37 (±24)	3	-1.69	0.831
2,3-Butanedione	46 (±13)	10	4 (±7)	3	-1.87	0.722
1-Hexanol	150 (±16)	10	111 (±27)	3	-1.11	0.958
(RS)-Linalool	50 (±6)	1	32 (±8)	4	-1.39	0.955
(E)-2-Hexenal	66 (±10)	10	23 (±12)	3	-2.03	0.634
ab6B Spontaneous activity	28 (±3)	14	8 (±3)	8	-3.3	0.01
Nepetalactol	-1 (±13)	3	-5 (± 3)	3	0.45	1
Sulcatone	-18 (±6)	10	-7 (±7)	3	0.69	1
(RS)- 1-Octen-3-ol	-3 (±9)	10	25 (±16)	3	1.53	0.975
Pentyl acetate	-20 (±5)	10	0 (±9)	3	1.69	0.944
Paraffin oil	-9 (±3)	14	1 (±3)	8	1.47	0.975
Methyl salicylate	-7 (±4)	10	-3 (±6)	3	0.76	1
Isoamyl acetate	-8 (±8)	8	4 (±18)	3	0.41	1
Geranyl acetate	-16 (±5)	10	-7 (±4)	3	1.02	0.998
Ethyl lactate	-5 (±4)	8	58 (±32)	5	1.92	0.802
Ethyl butanoate	-16 (±7)	10	-7 (±4)	3	0.51	1
Ethyl benzoate	-5 (±6)	8	-5 (±8)	3	0.1	1
Ethyl acetate	-9 (±7)	10	-6 (±4)	3	0.25	1
Diethyl succinate			0	1		
CO_2 (Breath)	-4 (±4)	8	-1 (±4)	3	0.92	0.999
Beta-cyclocitral	-15 (±3)	10	-7 (±6)	3	1.11	0.997
Benzaldehyde	-11 (±3)	1	16 (±9)	4	1.64	0.986
Alpha-Terpineol	-10 (±0)	1	3 (±3)	4	1.88	0.972
Acetophenone		0	2	1		
2-Phenylethanol	-13 (±1)	1	19 (±8)	4	1.85	0.972
2-Heptanone	-16 (±5)	10	-3 (±5)	3	1.28	0.992
2,3-Butanedione	-12 (±6)	10	-1 (±8)	3	0.51	1
1-Hexanol	-14 (±7)	10	2 (±12)	3	1.37	0.982
(RS)-Linalool	-22 (±2)	1	4 (±4)	4	1.85	0.972
(E)-2-Hexenal	-9 (±5)	10	3 (±11)	3	1.28	0.993
ab7A Spontaneous activity	15 (±2)	20	14 (±3)	11	-0.12	1
Nepetalactol	153 (±31)	3	51 (± 21)	3	-1.99	0.85
Sulcatone	178 (±15)	14	112 (±16)	5	-1.95	0.684
(RS)- 1-Octen-3-ol	29 (±6)	13	68 (±22)	5	1.62	0.85
Pentyl acetate	30 (±7)	13	67 (±14)	5	2.18	0.477
Paraffin oil	9 (±4)	20	12 (±5)	11	0.78	0.995
Methyl salicylate	21 (±5)	13	0 (±4)	5	-2.27	0.406

Isoamyl acetate	40 (±4)	12	149 (±8)	5	3.21	0.007
Geranyl acetate	19 (±7)	13	5 (±6)	5	-1.11	0.975
Ethyl lactate	149 (±15)	11	75 (±39)	5	-3.16	0.009
Ethyl butanoate	47 (±5)	13	26 (±12)	5	-1.85	0.726
Ethyl benzoate	35 (±5)	11	71 (±25)	5	1.06	0.977
Ethyl acetate	21 (±5)	13	2 (±6)	5	-1.99	0.65
Diethyl succinate	6	1				
CO_2 (Breath)	10 (±5)	12	0 (±4)	5	-1.29	0.947
Beta-cyclocitral	17 (±4)	13	6 (±7)	5	-1.34	0.94
Benzaldehyde	10 (±6)	4	16 (±3)	3	0.71	0.999
Alpha-Terpineol	17 (±9)	4	45 (±9)	3	1.77	0.85
Acetophenone	54	1	58	1		
2-Phenylethanol	34 (±3)	5	21 (±3)	3	-1.65	0.85
2-Heptanone	37 (±6)	13	112 (±19)	5	3.06	0.018
2,3-Butanedione	30 (±9)	13	-6 (±9)	5	-2.27	0.406
1-Hexanol	41 (±7)	13	50 (±33)	5	-0.6	0.999
(RS)-Linalool	14 (±8)	4	119 (±15)	3	2.12	0.692
(E)-2-Hexenal	50 (±6)	13	22 (±21)	5	-1.67	0.85
ab7B Spontaneous activity	17 (±2)	20	4 (±1)	11	-3.75	0.001
Nepetalactol	34 (±4)	3	24 (± 5)	3	-1.09	0.994
Sulcatone	11 (±8)	14	7 (±7)	5	0.14	1
(RS)- 1-Octen-3-ol	142 (±10)	13	0 (±5)	5	-3.26	0.002
Pentyl acetate	170 (±9)	13	1 (±2)	5	-3.24	0.004
Paraffin oil	13 (±3)	20	10 (±4)	11	-0.85	0.994
Methyl salicylate	34 (±11)	13	-2 (±3)	5	-3.07	0.015
Isoamyl acetate	162 (±10)	12	22 (±5)	5	-3.21	0.005
Geranyl acetate	83 (±13)	13	-4 (±3)	5	-2.87	0.037
Ethyl lactate	47 (±11)	11	7 (±7)	5	0.53	1
Ethyl butanoate	116 (±12)	13	19 (±8)	5	-3.2	0.005
Ethyl benzoate	102 (±16)	11	1 (±2)	5	-3.17	0.006
Ethyl acetate	35 (±8)	13	0 (±2)	5	-2.74	0.058
Diethyl succinate	8	1				
CO_2 (Breath)	6 (±2)	12	-2 (±2)	5	-2.44	0.176
Beta-cyclocitral	22 (±5)	13	0 (±3)	5	-2.46	0.155
Benzaldehyde	29 (±8)	4	1 (±1)	3	-2.14	0.506
Alpha-Terpineol	70 (±26)	4	6 (±2)	3	-1.07	0.99
Acetophenone	34	1	42	1		
2-Phenylethanol	12 (±10)	5	15 (±2)	3	0.15	1
2-Heptanone	145 (±12)	13	18 (±14)	5	-3.24	0.004
2,3-Butanedione	42 (±10)	13	-1 (±3)	5	-2.83	0.043
1-Hexanol	164 (±8)	13	10 (±6)	5	-3.24	0.004
(RS)-Linalool	135 (±28)	4	7 (±2)	3	-2.14	0.314
(E)-2-Hexenal	59 (±9)	13	-2 (±2)	5	-3.24	0.004
ab8A Spontaneous activity	7 (±1)	23	6 (±1)	7	0.58	1
Nepetalactol	8 (±4)	2	6 (± 6)	2	-0.41	1
Sulcatone	67 (±10)	18	37 (±10)	5	-1.45	0.985

(RS)- 1-Octen-3-ol	27 (±6)	18	16 (±7)	5	-1.24	0.997
Pentyl acetate	61 (±10)	18	57 (±17)	5	-0.15	1
Paraffin oil	6 (±2)	23	3 (±2)	7	-0.3	1
Methyl salicylate	9 (±3)	18	19 (±4)	4	1.28	0.997
Isoamyl acetate	81 (±12)	15	67 (±12)	5	-0.61	1
Geranyl acetate	26 (±6)	18	22 (±8)	5	0.04	1
Ethyl lactate	50 (±6)	13	28 (±8)	2	-1.36	0.997
Ethyl butanoate	143 (±10)	18	185 (±19)	5	1.68	0.938
Ethyl benzoate	19 (±4)	13	12 (±3)	5	-1.04	1
Ethyl acetate	32 (±7)	18	26 (±17)	5	-0.9	1
CO_2 (Breath)	5 (±3)	13	2 (±3)	5	-0.55	1
Beta-cyclocitral	4 (±3)	18	0 (±1)	5	-0.6	1
Benzaldehyde	11 (±14)	4	21 (±4)	3	1.06	1
Alpha-Terpineol	13 (±13)	4	-4 (±3)	3	-1.08	1
2-Phenylethanol	9 (±10)	4	-4 (±2)	3	-1.25	0.998
2-Heptanone	65 (±10)	18	64 (±20)	5	0.48	1
2,3-Butanedione	63 (±8)	18	45 (±8)	5	-0.82	1
1-Hexanol	94 (±9)	18	55 (±14)	4	-1.83	0.865
(RS)-Linalool	1 (±2)	3	-1 (±2)	3	-0.44	1
(E)-2-Hexenal	19 (±5)	18	42 (±16)	5	1.64	0.947
ab8B Spontaneous activity	9 (±2)	23	5 (±2)	7	-1.19	0.995
Nepetalactol	6 (±6)	2	10 (± 4)	2	0.77	1
Sulcatone	17 (±4)	18	19 (±7)	5	0.45	1
(RS)- 1-Octen-3-ol	43 (±6)	18	35 (±5)	5	-1.34	0.986
Pentyl acetate	47 (±9)	18	52 (±14)	5	0.15	1
Paraffin oil	3 (±2)	23	-2 (±1)	7	-2.03	0.627
Methyl salicylate	6 (±4)	18	8 (±4)	4	0.85	1
Isoamyl acetate	95 (±14)	15	85 (±11)	5	-0.65	1
Geranyl acetate	19 (±5)	18	6 (±5)	5	-0.9	1
Ethyl lactate	59 (±10)	13	53 (±47)	2	0	1
Ethyl butanoate	36 (±7)	18	39 (±4)	5	-0.49	1
Ethyl benzoate	21 (±6)	13	52 (±4)	5	2.82	0.073
Ethyl acetate	43 (±11)	18	21 (±9)	5	-0.63	1
CO_2 (Breath)	2 (±3)	13	1 (±3)	5	0.2	1
Beta-cyclocitral	5 (±3)	18	2 (±3)	5	-0.26	1
Benzaldehyde	36 (±10)	4	-1 (±1)	3	-2.16	0.726
Alpha-Terpineol	-8 (±3)	4	-1 (±1)	3	2.16	0.516
2-Phenylethanol	2 (±2)	4	1 (±1)	3	-0.19	1
2-Heptanone	69 (±10)	18	27 (±7)	5	-2.09	0.584
2,3-Butanedione	50 (±9)	18	99 (±17)	5	2.31	0.386
1-Hexanol	35 (±6)	18	31 (±15)	4	-0.94	1
(RS)-Linalool	-14 (±4)	3	-1 (±1)	3	2.02	0.891
(E)-2-Hexenal	43 (±6)	18	7 (±7)	5	-2.69	0.118
abXA Spontaneous activity	9 (± 1)	39				
Nepetalactol	27 (± 7)	11				
Sulcatone	149 (± 7)	22				

(RS)- 1-Octen-3-ol	119 (± 8)	22
Pentyl acetate	71 (± 6)	22
Paraffin oil	2 (± 1)	39
Nerol	157 (± 27)	6
Methyl salicylate	3 (± 2)	22
Isoamyl acetate	69 (± 6)	20
Geranyl acetate	41 (± 6)	22
Ethyl lactate	23 (± 3)	20
Ethyl butanoate	39 (± 3)	22
Ethyl benzoate	52 (± 4)	19
Ethyl acetate	8 (± 2)	22
Diethyl succinate	67 (± 13)	7
CO_2 (Breath)	3 (± 2)	20
Beta-cyclocitral	7 (± 2)	22
Benzaldehyde	25 (± 5)	4
Alpha-Terpineol	15 (± 4)	4
Acetophenone	6 (± 3)	7
2-Phenylethanol	17 (± 4)	5
2-Heptanone	69 (± 6)	22
2,3-Butanedione	12 (± 3)	22
1-Hexanol	57 (± 3)	22
(RS)-Linalool	183 (± 8)	4
(E)-2-Hexenal	30 (± 3)	22
abXB Spontaneous activity	9 (± 1)	39
Nepetalactol	93 (± 16)	11
Sulcatone	5 (± 5)	22
(RS)- 1-Octen-3-ol	0 (± 2)	22
Pentyl acetate	8 (± 6)	22
Paraffin oil	-3 (± 1)	39
Nerol	0 (± 3)	6
Methyl salicylate	0 (± 2)	22
Isoamyl acetate	10 (± 8)	20
Geranyl acetate	1 (± 3)	22
Ethyl lactate	9 (± 3)	20
Ethyl butanoate	0 (± 3)	22
Ethyl benzoate	1 (± 3)	19
Ethyl acetate	-1 (± 2)	22
Diethyl succinate	0 (± 4)	7
CO_2 (Breath)	-2 (± 2)	20
Beta-cyclocitral	21 (± 4)	22
Benzaldehyde	4 (± 2)	4
Alpha-Terpineol	-1 (± 2)	4
Acetophenone	36 (± 3)	7
2-Phenylethanol	5 (± 4)	5
2-Heptanone	9 (± 5)	22
2,3-Butanedione	-1 (± 2)	22

1-Hexanol	18 (± 3)	22		
(RS)-Linalool	1 (± 2)	4		
(E)-2-Hexenal	-1 (± 2)	22		
abYA Spontaneous activity			5 (± 1)	14
Nepetalactol			3 (± 4)	7
Sulcatone			97 (± 10)	6
(RS)- 1-Octen-3-ol			38 (± 5)	6
Pentyl acetate			65 (± 9)	5
Paraffin oil			0 (± 1)	14
Nerol			9 (± 5)	4
Methyl salicylate			5 (± 2)	6
Isoamyl acetate			67 (± 8)	5
Geranyl acetate			2 (± 2)	5
Ethyl lactate			2 (± 5)	5
Ethyl butanoate			44 (± 3)	6
Ethyl benzoate			163 (± 10)	9
Ethyl acetate			0 (± 2)	5
Diethyl succinate			42	1
CO_2 (Breath)			-1 (± 2)	5
Beta-cyclocitral			0 (± 2)	5
Benzaldehyde			18 (± 8)	3
Alpha-Terpineol			-3 (± 2)	3
Acetophenone			41 (± 13)	3
2-Phenylethanol			40 (± 24)	4
2-Heptanone			41 (± 3)	6
2,3-Butanedione			0 (± 2)	5
1-Hexanol			22 (± 8)	5
(RS)-Linalool			15 (± 6)	4
(E)-2-Hexenal			9 (± 2)	6
abYB Spontaneous activity			2 (± 1)	14
Nepetalactol			31 (± 8)	7
Sulcatone			2 (± 3)	6
(RS)- 1-Octen-3-ol			3 (± 4)	6
Pentyl acetate			0 (± 1)	5
Paraffin oil			0 (± 1)	14
Nerol			5 (± 1)	4
Methyl salicylate			0 (± 2)	6
Isoamyl acetate			2 (± 3)	5
Geranyl acetate			3 (± 2)	5
Ethyl lactate			0 (± 2)	5
Ethyl butanoate			3 (± 2)	6
Ethyl benzoate			7 (± 4)	9
Ethyl acetate			-2 (± 1)	5
Diethyl succinate			0	1
CO_2 (Breath)			0 (± 1)	5
Beta-cyclocitral			16 (± 5)	5

Benzaldehyde			0 (± 2)	3		
Alpha-Terpineol			-3 (± 1)	3		
Acetophenone			20 (± 5)	3		
2-Phenylethanol			-3 (± 1)	4		
2-Heptanone			-1 (± 2)	6		
2,3-Butanedione			-1 (± 0)	5		
1-Hexanol			5 (± 3)	5		
(RS)-Linalool			2 (± 2)	4		
(E)-2-Hexenal			0 (± 2)	6		
pb1A Spontaneous activity	18 (±3)	10				
Fenchone	24 (±4)	10	21 (±3)	8	-0.27	1
Cyclohexanone	47 (±7)	10	128 (±11)	8	3.47	0.002
4-Propylphenol	5 (±3)	10	9 (±2)	8	0.95	1
4-Methylphenol	11 (±5)	10	14 (±5)	8	0.22	1
3-Octanol	19 (±3)	10	30 (±4)	8	1.87	0.782
Paraffin oil	6 (±3)	10	4 (±2)	8	-0.63	1
Isoamyl acetate	17 (±4)	10	253 (±10)	8	3.56	0.001
Ethyl acetate	248 (±10)	10	286 (±7)	8	2.45	0.257
Benzaldehyde	19 (±4)	10	28 (±3)	8	1.65	0.912
2-Heptanone	170 (±13)	10	274 (±7)	8	3.56	0.001
(E)-2-Hexenal	131 (±13)	10	271 (±8)	8	3.56	0.001
pb1B Spontaneous activity	44 (±4)	10				
Fenchone	-12 (±5)	10	-2 (±1)	8	1.35	0.99
Cyclohexanone	-10 (±10)	10	-2 (±1)	8	1.56	0.955
4-Propylphenol	57 (±21)	10	76 (±15)	8	1.11	0.999
4-Methylphenol	174 (±20)	10	213 (±15)	8	0.89	1
3-Octanol	-6 (±4)	10	-3 (±1)	8	1.7	0.907
Paraffin oil	-4 (±3)	10	1 (±1)	8	1.06	0.999
Isoamyl acetate	-5 (±2)	10	4 (±3)	8	2.41	0.314
Ethyl acetate	-35 (±3)	10	1 (±1)	8	3.57	0.001
Benzaldehyde	3 (±3)	10	1 (±2)	8	-0.09	1
2-Heptanone	-32 (±5)	10	2 (±2)	8	3.39	0.005
(E)-2-Hexenal	-22 (±10)	10	1 (±1)	8	2.14	0.551
pb2A Spontaneous activity	24 (±3)	10				
Fenchone	257 (±8)	10	254 (±10)	8	-0.22	1
Cyclohexanone	192 (±10)	10	214 (±7)	8	1.2	0.998
4-Propylphenol	-1 (±2)	10	4 (±2)	8	1.71	0.895
4-Methylphenol	4 (±6)	10	29 (±3)	8	2.54	0.211
3-Octanol	28 (±4)	10	14 (±2)	8	-2.24	0.459
Paraffin oil	1 (±3)	10	3 (±2)	8	0.85	1
Isoamyl acetate	31 (±4)	10	31 (±3)	8	-0.4	1
Ethyl acetate	103 (±15)	10	122 (±19)	8	0.76	1
Benzaldehyde	116 (±10)	10	35 (±2)	8	-3.56	<0.001
2-Heptanone	13 (±3)	10	16 (±2)	8	0.54	1
(E)-2-Hexenal	15 (±5)	10	37 (±3)	8	2.68	0.137
pb2B Spontaneous activity	44 (±2)	10				

Fenchone	-36 (±3)	10	-19 (±3)	8	3.03	0.03
Cyclohexanone	-35 (±3)	10	-17 (±3)	8	3.12	0.023
4-Propylphenol	-1 (±4)	10	-1 (±3)	8	-0.13	1
4-Methylphenol	133 (±11)	10	202 (±14)	8	2.84	0.067
3-Octanol	-12 (±2)	10	-22 (±2)	8	-2.36	0.328
Paraffin oil	-3 (±4)	10	1 (±1)	8	1	1
Isoamyl acetate	-9 (±3)	10	-12 (±2)	8	-0.85	1
Ethyl acetate	-23 (±4)	10	-14 (±2)	8	2.24	0.435
Benzaldehyde	-4 (±3)	10	53 (±5)	8	3.57	0.001
2-Heptanone	-7 (±4)	10	-10 (±2)	8	-0.99	1
(E)-2-Hexenal	-4 (±4)	10	-8 (±2)	8	-1.61	0.932
pb3A Spontaneous activity	39 (±2)	10				
Fenchone	0 (±3)	10	1 (±1)	8	0.77	1
Cyclohexanone	3 (±4)	10	7 (±2)	8	0.76	1
4-Propylphenol	-5 (±4)	10	1 (±1)	8	1.07	1
4-Methylphenol	-7 (±2)	10	0 (±1)	8	2.48	0.275
3-Octanol	21 (±4)	10	40 (±5)	8	2.41	0.296
Paraffin oil	-3 (±4)	10	1 (±1)	8	0.9	1
Isoamyl acetate	9 (±3)	10	24 (±4)	8	2.31	0.37
Ethyl acetate	2 (±3)	10	28 (±4)	8	3.56	<0.001
Benzaldehyde	-5 (±3)	10	1 (±1)	8	1.03	1
2-Heptanone	6 (±5)	10	30 (±4)	8	2.68	0.135
(E)-2-Hexenal	7 (±5)	10	-1 (±1)	8	-1.08	1
pb3B Spontaneous activity	14 (±2)	10				
Fenchone	4 (±3)	10	3 (±2)	8	-0.54	1
Cyclohexanone	1 (±3)	10	3 (±2)	8	0.54	1
4-Propylphenol	1 (±2)	10	2 (±1)	8	0.68	1
4-Methylphenol	1 (±2)	10	-3 (±2)	8	-1.4	0.988
3-Octanol	8 (±3)	10	76 (±8)	8	3.56	0.001
Paraffin oil	-1 (±2)	10	5 (±3)	8	1.52	0.972
Isoamyl acetate	100 (±12)	10	112 (±9)	8	0.58	1
Ethyl acetate	49 (±6)	10	38 (±4)	8	-1.2	0.998
Benzaldehyde	12 (±3)	10	34 (±3)	8	3.34	0.006
2-Heptanone	143 (±15)	10	188 (±13)	8	2.13	0.572
(E)-2-Hexenal	24 (±4)	10	35 (±4)	8	1.87	0.806

10.9 APPENDIX 10. COMPARISON OF ORN RESPONSES OF AB3-HIGH AND AB3-LOW LINES IN D. SUZUKII

Table 10-16 ab3-high and ab3-low ORN responses to fruit headspaces

Mean (± SEM) impulses/s during a 0.5 s stimulus with headspace of ripe whole fruits. Data originated for chapter 3. Comparison of responses between lines using a Wilcoxon signed-ranked test followed by a Holm-Sidak correction for multiple comparisons. There were no significant differences.

		ab3-high		ab3-low			
ORN	**Headspace**	**Response**	**N**	**Response**	**N**	**Z stat**	**P value**
ab1A	Strawberry	79 (±22)	4	63 (±6)	5	1.358	0.802
	Raspberry	44 (±12)	4	43 (±4)	5	1.257	0.802
	Blueberry	44 (±3)	4	45 (±5)	5	0.313	0.975
	Grape	8 (±2)	4	19 (±4)	5	-1.379	0.802
	Orange	40 (±14)	4	32 (±8)	5	0.733	0.938
	Tomato	30 (±9)	4	39 (±9)	5	-0.105	0.984
	Control	53 (±11)	4	54 (±6)	5	2.095	0.277
	Baseline	36 (±7)	4	27 (±5)	5	-0.733	0.938
ab1B	Strawberry	26 (±8)	4	31 (±9)	5	0.952	0.956
	Raspberry	10 (±5)	4	9 (±3)	5	0.838	0.956
	Blueberry	8 (±3)	4	25 (±11)	5	0.210	0.986
	Grape	36 (±3)	4	13 (±10)	5	0.105	0.986
	Orange	21 (±5)	4	40 (±18)	5	-0.419	0.982
	Tomato	35 (±5)	4	35 (±3)	5	-0.629	0.971
	Control	11 (±2)	4	18 (±5)	5	-1.362	0.787
	Baseline	34 (±6)	4	24 (±4)	5	-1.576	0.709
ab1C	Strawberry	16 (±3)	4	19 (±4)	5	-1.152	0.740
	Raspberry	13 (±6)	4	13 (±5)	5	0.849	0.821
	Blueberry	22 (±4)	4	17 (±6)	5	-0.210	0.954
	Grape	9 (±4)	4	12 (±5)	5	0.315	0.954
	Orange	36 (±4)	4	21 (±3)	5	1.362	0.685
	Tomato	21 (±4)	4	34 (±6)	5	1.370	0.684
	Control	34 (±5)	4	22 (±2)	5	1.571	0.637
	Baseline	4 (±2)	4	16 (±3)	5	1.997	0.408
ab1D	Strawberry	16 (±1)	4	18 (±4)	5	0.868	0.935
	Raspberry	40 (±2)	4	42 (±1)	5	-0.900	0.935
	Blueberry	33 (±4)	4	33 (±7)	5	-1.892	0.405
	Grape	25 (±5)	4	16 (±3)	5	-1.375	0.779
	Orange	19 (±2)	4	14 (±1)	5	-0.967	0.935
	Tomato	6 (±3)	4	13 (±3)	5	-0.108	1.000
	Control	11 (±5)	4	19 (±6)	5	-2.214	0.175
	Baseline	32 (±10)	4	23 (±14)	5	-0.636	0.935
ab2A	Strawberry	24 (±5)	6	35 (±9)	5	-0.091	1.000
	Raspberry	39 (±7)	6	40 (±2)	5	1.461	0.791
	Blueberry	13 (±4)	6	24 (±6)	5	0.183	1.000
	Grape	24 (±5)	6	16 (±2)	5	0.550	0.984
	Orange	15 (±3)	6	13 (±4)	5	-1.009	0.928
	Tomato	9 (±3)	6	17 (±3)	5	-0.839	0.949
	Control	12 (±2)	6	9 (±1)	5	-0.093	1.000
	Baseline	12 (±2)	6	9 (±2)	5	-1.376	0.791

ab2B	Strawberry	54 (±6)	6	66 (±5)	5	-0.550	0.968
	Raspberry	36 (±9)	6	33 (±6)	5	-2.401	0.115
	Blueberry	45 (±4)	6	40 (±7)	5	-0.730	0.968
	Grape	11 (±4)	6	21 (±5)	5	-1.379	0.736
	Orange	27 (±4)	6	24 (±6)	5	0.740	0.968
	Tomato	32 (±4)	6	40 (±3)	5	-0.184	0.991
	Control	25 (±5)	6	36 (±4)	5	-1.502	0.678
	Baseline	27 (±7)	6	14 (±2)	5	0.000	1.000
ab3A	Strawberry	79 (±8)	8	90 (±19)	7	0.116	0.988
	Raspberry	4 (±3)	8	7 (±4)	7	0.988	0.951
	Blueberry	10 (±5)	8	23 (±9)	7	-0.869	0.951
	Grape	37 (±6)	8	20 (±15)	7	-1.509	0.712
	Orange	26 (±6)	8	37 (±3)	7	-0.869	0.951
	Tomato	35 (±7)	8	38 (±2)	7	-0.930	0.951
	Control	12 (±2)	8	14 (±4)	7	-0.174	0.988
	Baseline	33 (±3)	8	37 (±5)	7	0.699	0.951
ab3B	Strawberry	16 (±2)	8	14 (±2)	7	0.464	0.851
	Raspberry	13 (±6)	8	13 (±3)	7	2.143	0.226
	Blueberry	18 (±4)	8	16 (±6)	7	0.869	0.851
	Grape	7 (±3)	8	3 (±2)	7	1.425	0.704
	Orange	56 (±6)	8	52 (±4)	7	-0.812	0.851
	Tomato	26 (±6)	8	31 (±7)	7	0.929	0.851
	Control	44 (±5)	8	32 (±7)	7	1.454	0.704
	Baseline	17 (±5)	8	15 (±1)	7	1.450	0.704
ab4A	Strawberry	28 (±9)	5	48 (±14)	5	-1.410	0.843
	Raspberry	37 (±5)	5	31 (±7)	5	0.522	0.991
	Blueberry	62 (±14)	5	70 (±9)	5	0.000	1.000
	Grape	24 (±5)	5	27 (±5)	5	-0.313	0.996
	Orange	20 (±5)	5	23 (±4)	5	0.000	1.000
	Tomato	19 (±10)	5	14 (±3)	5	0.731	0.991
	Control	21 (±10)	5	26 (±8)	5	1.185	0.905
	Baseline	36 (±8)	5	15 (±8)	5	0.632	0.991
ab4B	Strawberry	27 (±4)	5	32 (±6)	5	-0.422	0.999
	Raspberry	23 (±10)	5	35 (±5)	5	-0.427	0.999
	Blueberry	15 (±4)	5	14 (±3)	5	-0.106	0.999
	Grape	31 (±3)	5	36 (±7)	5	-0.535	0.999
	Orange	16 (±4)	5	17 (±3)	5	0.105	0.999
	Tomato	7 (±2)	5	11 (±3)	5	1.606	0.767
	Control	87 (±21)	5	139 (±42)	5	-1.601	0.767
	Baseline	6 (±2)	5	5 (±4)	5	-0.210	0.999
ab5A	Strawberry	150 (±15)	5	120 (±17)	3	-1.518	0.826
	Raspberry	65 (±10)	5	53 (±7)	3	-1.050	0.867
	Blueberry	50 (±3)	5	43 (±7)	3	-1.200	0.867
	Grape	1 (±1)	5	5 (±3)	3	-1.200	0.867
	Orange	122 (±9)	5	99 (±14)	3	-0.447	0.954
	Tomato	5 (±2)	5	17 (±4)	3	-1.350	0.843
	Control	66 (±6)	5	58 (±6)	3	-1.207	0.867
	Baseline	71 (±20)	5	33 (±8)	3	-0.458	0.954
ab5B	Strawberry	34 (±6)	5	39 (±16)	3	1.200	0.822
	Raspberry	4 (±2)	5	5 (±3)	3	1.342	0.822
	Blueberry	11 (±4)	5	23 (±10)	3	1.640	0.709
	Grape	36 (±8)	5	21 (±13)	3	1.043	0.822
	Orange	18 (±3)	5	30 (±10)	3	1.640	0.709

	Tomato	38 (±13)	5	32 (±4)	3	1.043	0.822
	Control	13 (±4)	5	21 (±4)	3	0.447	0.822
	Baseline	41 (±7)	5	47 (±14)	3	1.200	0.822
ab6A	Strawberry	15 (±2)	3	19 (±6)	2	-1.732	0.832
	Raspberry	16 (±5)	3	8 (±3)	2	-1.155	0.953
	Blueberry	27 (±3)	3	19 (±2)	2	-0.577	0.953
	Grape	11 (±8)	3	3 (±1)	2	-1.155	0.953
	Orange	15 (±4)	3	18 (±5)	2	-0.889	0.953
	Tomato	15 (±4)	3	25 (±5)	2	-1.155	0.953
	Control	28 (±2)	3	20 (±2)	2	-1.155	0.953
	Baseline	10 (±3)	3	12 (±3)	2	-1.481	0.918
ab6B	Strawberry	13 (±3)	3	19 (±2)	2	-0.577	1.000
	Raspberry	37 (±3)	3	35 (±3)	2	0.296	1.000
	Blueberry	23 (±3)	3	20 (±3)	2	0.000	1.000
	Grape	21 (±3)	3	15 (±3)	2	-0.577	1.000
	Orange	16 (±2)	3	14 (±3)	2	-0.577	1.000
	Tomato	7 (±3)	3	10 (±5)	2	-0.296	1.000
	Control	9 (±6)	3	16 (±9)	2	0.000	1.000
	Baseline	31 (±9)	3	15 (±9)	2	0.592	0.999
ab7A	Strawberry	13 (±2)	6	19 (±3)	5	-0.913	0.939
	Raspberry	36 (±10)	6	34 (±6)	5	-1.921	0.382
	Blueberry	11 (±4)	6	18 (±7)	5	-1.388	0.785
	Grape	18 (±3)	6	14 (±2)	5	-0.276	0.943
	Orange	15 (±4)	6	14 (±1)	5	-0.551	0.943
	Tomato	10 (±2)	6	12 (±3)	5	0.642	0.943
	Control	9 (±2)	6	12 (±2)	5	-1.287	0.785
	Baseline	9 (±4)	6	3 (±2)	5	-0.734	0.943
ab7B	Strawberry	148 (±26)	6	105 (±18)	5	0.917	0.917
	Raspberry	38 (±9)	6	32 (±7)	5	-0.091	0.970
	Blueberry	38 (±8)	6	51 (±4)	5	1.108	0.917
	Grape	6 (±2)	6	4 (±2)	5	-0.555	0.917
	Orange	149 (±21)	6	150 (±26)	5	-0.919	0.917
	Tomato	16 (±3)	6	27 (±11)	5	-1.095	0.917
	Control	133 (±16)	6	135 (±11)	5	1.481	0.795
	Baseline	49 (±15)	6	38 (±5)	5	1.108	0.917
ab8A	Strawberry	30 (±2)	6	38 (±5)	4	-0.216	1.000
	Raspberry	8 (±3)	6	9 (±2)	4	-0.642	0.999
	Blueberry	24 (±5)	6	33 (±3)	4	-0.650	0.999
	Grape	35 (±5)	6	23 (±2)	4	0.650	0.999
	Orange	27 (±4)	6	39 (±1)	4	-0.107	1.000
	Tomato	39 (±4)	6	39 (±1)	4	-0.536	0.999
	Control	20 (±5)	6	38 (±12)	4	0.541	0.999
	Baseline	47 (±5)	6	40 (±3)	4	0.107	1.000
ab8B	Strawberry	34 (±7)	6	35 (±9)	4	-0.647	0.977
	Raspberry	29 (±7)	6	35 (±8)	4	1.287	0.844
	Blueberry	95 (±11)	6	108 (±19)	4	-0.432	0.977
	Grape	4 (±2)	6	3 (±1)	4	-0.863	0.955
	Orange	42 (±6)	6	45 (±6)	4	-1.324	0.844
	Tomato	22 (±5)	6	27 (±6)	4	0.322	0.977
	Control	40 (±3)	6	35 (±2)	4	-1.823	0.491
	Baseline	10 (±2)	6	12 (±4)	4	-0.548	0.977
abXA	Strawberry	24 (±3)	4	29 (±4)	3	-0.707	0.953
	Raspberry	40 (±4)	4	40 (±7)	3	1.620	0.778

	Blueberry	48 (±5)	4	52 (±5)	3	1.070	0.947
	Grape	26 (±6)	4	18 (±4)	3	0.354	0.953
	Orange	54 (±10)	4	60 (±23)	3	-1.061	0.953
	Tomato	8 (±2)	4	3 (±1)	3	0.535	0.953
	Control	8 (±4)	4	21 (±9)	3	1.101	0.953
	Baseline	32 (±9)	4	20 (±15)	3	-1.080	0.953
abXB	Strawberry	27 (±5)	4	39 (±9)	3	0.540	0.992
	Raspberry	31 (±6)	4	34 (±4)	3	0.370	0.992
	Blueberry	37 (±11)	4	29 (±10)	3	-0.535	0.992
	Grape	81 (±15)	4	105 (±10)	3	0.892	0.992
	Orange	16 (±4)	4	19 (±2)	3	0.000	1.000
	Tomato	10 (±3)	4	12 (±5)	3	-0.900	0.992
	Control	21 (±4)	4	18 (±5)	3	1.080	0.992
	Baseline	3 (±2)	4	5 (±2)	3	0.926	0.992

Table 10-17 ab3-high and ab3-low ORN responses to fruit volatiles

Mean (± SEM) impulses/s during a 0.5 s stimulus with 0.01% dose of chemical. Data originated for chapter 4. Comparison of responses between lines using a Wilcoxon signed-ranked test followed by a Holm-Sidak correction for multiple comparisons. There were no significant differences.

		ab3-high		**ab3-low**			
ORN	**Headspace**	**Response**	**N**	**Response**	**N**	***Z stat***	***P value***
ab1A	(Z)-3-Hexenal	20 (±1)	3	26 (±4)	2	1.481	1
	(Z)-3-Hexenol	31 (±6)	3	54 (±8)	2	1.732	1
	2-(ethoxyethoxy)-ethanol	36 (±7)	3	18	1	-1.342	1
	2-Heptanone	81 (±12)	3	78 (±8)	2	0	1
	4-Valerolactone	23 (±3)	3	28	1	0.943	1
	Alpha-Pinene	21 (±6)	3	26 (±18)	2	0	1
	Benzaldehyde	22 (±4)	3	22	1	-0.447	1
	Beta-cyclocitral	14 (±3)	3	24 (±2)	2	1.732	1
	Butyl acetate	11 (±11)	3	10 (±8)	2	0	1
	Ethyl butanoate	149 (±18)	3	139 (±5)	2	0	1
	Ethyl cyclopentane	23 (±3)	3	28	1	0.943	1
	Heptanal	23 (±3)	3	6	1	-1.342	1
	Hexyl acetate	26 (±3)	3	16 (±10)	2	-0.889	1
	Isoamyl acetate	23 (±6)	3	18 (±4)	2	-0.296	1
	Isobutyl acetate	85 (±11)	3	77 (±7)	2	-0.592	1
	Methyl hexanoate	8 (±8)	3	17 (±9)	2	0.577	1
	Nonanal	3 (±4)	3	28	1	1.342	1
	Paraffin oil	19 (±4)	13	14 (±6)	5	-1.009	1
	Pentyl acetate	13 (±3)	3	10 (±6)	2	-0.592	1
	p-ethylstyrene	27 (±3)	3	-4	1	-1.414	1
	Prenyl acetate	17 (±3)	3	8 (±6)	2	-1.155	1
	Spontaneous activity	19 (±1)	13	17 (±3)	5	-1.111	1
	Triacetin	17 (±1)	3	20 (±8)	2	0	1
ab1B	(Z)-3-Hexenal	0 (±2)	3	11 (±1)	2	1.777	0.975
	(Z)-3-Hexenol	5 (±3)	3	8 (±8)	2	0	1
	2-(ethoxyethoxy)-ethanol	12 (±9)	3	-6	1	-1.342	1

	2-Heptanone	9 (±7)	3	-7 (±1)	2	-1.777	0.999
	4-Valerolactone	7 (±8)	3	-4	1	-0.447	1
	Alpha-Pinene	6 (±4)	3	10 (±8)	2	0.577	1
	Benzaldehyde	11 (±8)	3	0	1	-0.447	1
	Beta-cyclocitral	9 (±4)	3	0 (±0)	2	-1.777	0.999
	Butyl acetate	3 (±4)	3	0 (±2)	2	-0.296	1
	Ethyl butanoate	15 (±9)	3	2 (±2)	2	-0.889	1
	Ethyl cyclopentane	7 (±8)	3	-4	1	-0.447	1
	Heptanal	9 (±5)	3	-4	1	-1.342	1
	Hexyl acetate	5 (±2)	3	-1 (±1)	2	-1.481	1
	Isoamyl acetate	5 (±1)	3	5 (±5)	2	0	1
	Isobutyl acetate	7 (±4)	3	3 (±1)	2	-0.577	1
	Methyl hexanoate	3 (±2)	3	7 (±9)	2	0.296	1
	Nonanal	3 (±3)	3	8	1	0.943	1
	Paraffin oil	4 (±2)	13	7 (±6)	5	-0.092	1
	Pentyl acetate	6 (±5)	3	-2 (±0)	2	-0.592	1
	p-ethylstyrene	10 (±8)	3	16	1	0.447	1
	Prenyl acetate	1 (±3)	3	-5 (±1)	2	-1.481	1
	Spontaneous activity	5 (±2)	13	12 (±2)	5	2.114	0.761
	Triacetin	3 (±5)	3	-2 (±2)	2	-0.577	1
ab1C	(Z)-3-Hexenal	18 (±3)	3	7 (±1)	2	-1.732	0.999
	(Z)-3-Hexenol	5 (±11)	3	-2 (±8)	2	-0.296	1
	2-(ethoxyethoxy)-ethanol	10 (±3)	3	-4	1	-1.342	1
	2-Heptanone	7 (±14)	3	-3 (±9)	2	-0.577	1
	4-Valerolactone	3 (±2)	3	4	1	0	1
	Alpha-Pinene	17 (±5)	3	1 (±3)	2	-1.732	0.999
	Benzaldehyde	8 (±3)	3	4	1	-0.943	1
	Beta-cyclocitral	16 (±2)	3	6 (±4)	2	-1.732	0.999
	Butyl acetate	17 (±2)	3	-1 (±9)	2	-1.732	0.999
	Ethyl butanoate	3 (±8)	3	-10 (±10)	2	-1.155	1
	Ethyl cyclopentane	3 (±2)	3	4	1	0	1
	Heptanal	5 (±2)	3	12	1	1.342	1
	Hexyl acetate	17 (±3)	3	0 (±4)	2	-1.777	0.999
	Isoamyl acetate	22 (±4)	3	3 (±7)	2	-1.732	0.999
	Isobutyl acetate	8 (±3)	3	-5 (±9)	2	-1.481	1
	Methyl hexanoate	17 (±2)	3	3 (±7)	2	-1.732	0.999
	Nonanal	5 (±3)	3	10	1	0.943	1
	Paraffin oil	8 (±2)	13	10 (±3)	5	0.46	1
	Pentyl acetate	3 (±10)	3	2 (±6)	2	-0.577	1
	p-ethylstyrene	4 (±3)	3	-12	1	-1.342	1
	Prenyl acetate	16 (±4)	3	-2 (±10)	2	-1.777	0.975
	Spontaneous activity	14 (±3)	13	21 (±2)	5	1.834	0.931
	Triacetin	18 (±3)	3	11 (±1)	2	-1.732	0.999
ab1D	(Z)-3-Hexenal	-1 (±1)	3	2 (±2)	2	1.291	1
	(Z)-3-Hexenol	-1 (±2)	3	3 (±11)	2	0	1
	2-(ethoxyethoxy)-ethanol	0 (±2)	3	-4	1	-1	1
	2-Heptanone	-3 (±3)	3	-5 (±3)	2	-0.913	1
	4-Valerolactone	8 (±8)	3	8	1	0.447	1
	Alpha-Pinene	-1 (±1)	3	3 (±7)	2	0.304	1
	Benzaldehyde	2 (±3)	3	-6	1	-1.342	1
	Beta-cyclocitral	-1 (±1)	3	5 (±7)	2	0.304	1

	Butyl acetate	-3 (±3)	3	-3 (±5)	2	0.304	1
	Ethyl butanoate	-3 (±3)	3	0 (±2)	2	0.592	1
	Ethyl cyclopentane	8 (±8)	3	8	1	0.447	1
	Heptanal	-1 (±1)	3	-4	1	-1.414	1
	Hexyl acetate	-1 (±1)	3	-2 (±0)	2	-1.333	1
	Isoamyl acetate	1 (±1)	3	0 (±2)	2	-0.304	1
	Isobutyl acetate	-3 (±3)	3	-5 (±3)	2	-0.913	1
	Methyl hexanoate	1 (±1)	3	1 (±1)	2	0	1
	Nonanal	2 (±3)	3	-6	1	-1.342	1
	Paraffin oil	0 (±1)	13	3 (±4)	5	0.373	1
	Pentyl acetate	1 (±1)	3	1 (±3)	2	0	1
	p-ethylstyrene	-2 (±5)	3	4	1	0.447	1
	Prenyl acetate	-2 (±2)	3	0 (±8)	2	0	1
	Spontaneous activity	4 (±2)	13	6 (±2)	5	0.749	1
	Triacetin	-3 (±3)	3	-2 (±0)	2	-0.609	1
ab3A	(Z)-3-Hexenal	15 (±7)	4	59 (±9)	2	1.852	0.994
	(Z)-3-Hexenol	54 (±16)	4	53 (±21)	2	0	1
	2-(ethoxyethoxy)-ethanol	24 (±7)	4	14 (±8)	2	-0.953	1
	2-Heptanone	113 (±8)	2	148 (±12)	2	1.852	0.994
	3-Hydroxy-2-butanone	36 (±10)	2	12 (±3)	3	-1.732	0.999
	4-Decalactone	15 (±7)	2	29 (±4)	3	1.732	0.999
	4-Valerolactone	29 (±4)	3	24 (±12)	2	0	1
	Alpha-Pinene	31 (±9)	4	47 (±11)	1	0.926	1
	Benzaldehyde	27 (±7)	3	15 (±3)	2	-1.155	1
	Beta-cyclocitral	124 (±8)	4	51 (±17)	1	-1.852	0.994
	Butyl acetate	145 (±14)	4	105 (±39)	1	-0.926	1
	DMNT	19 (±1)	2	23 (±15)	3	0.577	1
	Ethyl acetate	37 (±7)	2	43 (±12)	3	0.577	1
	Ethyl butanoate	128 (±17)	4	175 (±15)	1	1.389	0.999
	Ethyl cyclopentane	29 (±4)	3	24 (±12)	2	0	1
	Heptanal	23 (±1)	2	4 (±0)	2	-1.633	1
	Hexanoic acid	24 (±2)	2	27 (±12)	3	0.296	1
	Hexyl acetate	24 (±2)	4	34 (±6)	2	1.644	0.999
	Isoamyl acetate	126 (±7)	4	149 (±13)	2	1.389	0.999
	Isoamyl alcohol	24 (±0)	2	19 (±16)	3	0.592	1
	Isobutyl acetate	156 (±16)	3	186 (±8)	2	0.926	1
	Isopropyl butanoate	76 (±4)	2	101 (±34)	3	0.577	1
	Methyl butanoate	149 (±1)	2	159 (±10)	2	0.609	1
	Methyl hexanoate	74 (±11)	4	126 (±20)	2	1.852	0.994
	Methyl pentanoate	135 (±3)	2	143 (±11)	2	0.577	1
	Methyl-(E)-2-hexenoate	141 (±1)	2	101 (±37)	3	-0.577	1
	Myrcene	25 (±5)	2	31 (±3)	3	1.185	0.999
	n-Butyric acid	29 (±3)	2	19 (±12)	3	-0.577	1
	Nonanal	9 (±6)	3	8 (±4)	2	0	1
	Paraffin oil	26 (±4)	22	18 (±6)	7	-1.225	0.999
	Pentyl acetate	113 (±11)	4	163 (±11)	2	1.852	0.994
	p-ethylstyrene	22 (±3)	3	7 (±3)	2	-1.732	0.999
	Prenyl acetate	123 (±12)	4	174 (±14)	2	1.669	0.999
	Sec-butyl-butanoate	39 (±5)	2	74 (±6)	3	1.732	0.999
	Spontaneous activity	14 (±3)	22	16 (±2)	7	0.394	1
	Triacetin	11 (±8)	2	35 (±7)	2	1.879	0.994
ab3B	(Z)-3-Hexenal	9 (±11)	4	-5 (±1)	2	-1.174	1

(Z)-3-Hexenol	38 (±4)	4	44 (±16)	2	0	1
2-(ethoxyethoxy)-ethanol	11 (±4)	4	18 (±4)	2	1.174	1
2-Heptanone	175 (±16)	2	176 (±4)	2	0.705	1
3-Hydroxy-2-butanone	20 (±2)	2	11 (±2)	3	-1.732	0.999
4-Decalactone	21 (±7)	2	33 (±1)	3	1.777	0.999
4-Valerolactone	7 (±8)	3	26 (±6)	2	1.155	1
Alpha-Pinene	8 (±6)	4	0 (±2)	1	-0.926	1
Benzaldehyde	17 (±6)	3	10 (±8)	2	-1.155	1
Beta-cyclocitral	12 (±3)	4	4 (±6)	1	-1.192	1
Butyl acetate	109 (±17)	4	125 (±95)	1	0	1
DMNT	29 (±5)	2	22 (±9)	3	-0.577	1
Ethyl acetate	8 (±2)	2	10 (±2)	3	0.609	1
Ethyl butanoate	9 (±10)	4	-17 (±11)	1	-1.389	1
Ethyl cyclopentane	7 (±8)	3	26 (±6)	2	1.155	1
Heptanal	11 (±9)	2	21 (±1)	2	1.225	1
Hexanoic acid	27 (±5)	2	21 (±3)	3	-0.889	1
Hexyl acetate	6 (±7)	4	-2 (±2)	2	-0.926	1
Isoamyl acetate	16 (±11)	4	5 (±13)	2	-0.705	1
Isoamyl alcohol	5 (±1)	2	3 (±1)	3	-1.291	1
Isobutyl acetate	14 (±9)	3	-5 (±7)	2	-1.389	1
Isopropyl butanoate	25 (±1)	2	21 (±2)	3	-1.481	1
Methyl butanoate	12 (±2)	2	29 (±5)	2	1.732	0.999
Methyl hexanoate	7 (±8)	4	-2 (±8)	2	-0.463	1
Methyl pentanoate	12 (±4)	2	28 (±4)	2	1.777	0.977
Methyl-(E)-2-hexenoate	3 (±3)	2	5 (±2)	3	0.889	1
Myrcene	11 (±1)	2	7 (±3)	3	-0.592	1
n-Butyric acid	11 (±7)	2	7 (±4)	3	-0.577	1
Nonanal	-1 (±3)	3	13 (±1)	2	1.732	0.999
Paraffin oil	7 (±2)	22	12 (±4)	7	0.88	1
Pentyl acetate	39 (±3)	4	33 (±11)	2	-0.463	1
p-ethylstyrene	8 (±11)	3	18 (±4)	2	0.577	1
Prenyl acetate	13 (±13)	4	2 (±14)	2	-0.463	1
Sec-butyl-butanoate	6 (±0)	2	5 (±4)	3	0	1
Spontaneous activity	15 (±2)	22	11 (±4)	7	-1.084	1
Triacetin	15 (±15)	2	-6 (±0)	2	-1.879	0.993