Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Landscape Architecture, Horticulture and Crop Production Science

The behavioural response of *Drosophila suzukii* to fermentation products

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Degree Project • 30 hec
Hortonomprogrammet /Horticultural Science
Alnarp 2018

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Påverkan av fermenteringsprodukter på *Drosophila suzukiis* beteende

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Credits: 30 hec
Project level: A2E
Course Title: Master's Thesis Project in Biology
Course Code: EX0800
Subject: Biology

Programme: Hortonomprogrammet /Horticultural Science

Place of Publication: Alnarp
Year of Publication: 2018
Cover Art: Isabella Kleman
Online Publication: http://stud.epsilon.slu.se

Keywords: *Drosophila suzukii*, SWD, behaviour, yeast, fermentation, *Hanseniaspora uvarum*, monitoring

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Department of Plant Breeding

Abstract

Drosophila suzukii, or the Spotted Wing Drosophila (SWD), is an invasive fly that is a pest of soft fruits and berries. Unlike other *Drosophila* species, the females lay their eggs in ripening and ripe fruits, causing crop losses. In the last decade SWD has invaded North and South America and Europe and since 2014 the fly can also be found in Sweden. Despite preferring to oviposit in ripe fruit, *D. suzukii* is, like many other drosophilids, dependent on yeasts as a source of important nutrients. An especially strong connection has previously been seen to the yeast *Hanseniaspora uvarum*. In this work the behavioural effect of this yeast on *D. suzukii* was investigated. A larval assay was carried out to identify the most attractive single compounds from the headspace of *H. uvarum*. A blend of two of the more attractive compounds, ethyl acetate and acetoin, produced similar attraction levels as an *H. uvarum* liquid culture. Oviposition assays were carried out with real and artificial blueberries where mated female flies were given a choice between laying eggs in berries inoculated with undiluted, diluted or no *H. uvarum*. While no significant differences could be found between the treatments, it was noted that females were more likely to lay eggs if given access to undiluted yeast. Mating observations to investigate where matings where most likely to take place showed that a majority of the flies aggregated and mated on raspberries inoculated with *H. uvarum* compared to clean raspberries, fresh raspberry shoots and an *H. uvarum* culture the flies could smell but not feed on. Finally, a monitoring project was carried out in collaboration with hobby growers to investigate the current distribution of *D. suzukii* in Sweden. The results showed that *D. suzukii* is now present in most of southern Sweden, up to and including Stockholm, in both populated and rural areas.

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Introduction

The biology of *Drosophila suzukii*

Many *Drosophila* species depend on yeasts as their main food source, both in their larval and adult stages (Begon, 1982). Therefore, they tend to feed and reproduce on fermenting or rotting materials and cause no major harm to crop production.

However, *Drosophila suzukii,* the Spotted Wing Drosophila (SWD), has evolved a sclerotized and serrated ovipositor enabling the females to lay eggs in the undamaged ripe or ripening soft fruits and berries of a variety of species, many of them important crops (Atallah et al., 2014, Lee et al., 2011). One other species of *Drosophila* known to be able to lay eggs in undamaged berries is *D. subpulchrella*, but this species is not known for causing any damage in crops (Atallah et al., 2014). Together with the evolution of the ovipositor, *D. suzukii* has developed a preference for ovipositing in ripe fruit rather than the overripe preferred by closely related species (Karageorgi et al., 2017). SWD also has a short generation time, around 12 days under optimal conditions (Tochen et al., 2014), and has been reported to produce as many as 13 generations in a season (Kanzawa, 1939). More generations may potentially be possible in climates with a longer growing season. Like other *Drosophila* species, *D. suzukii* goes through 3 larval stages. Upon reaching the third instar and becoming ready to pupate the larvae will enter a wandering stage in which it stops feeding (Jakobs et al., 2017) and often exits the fruit to pupate in the soil (Woltz and Lee, 2017).

In *Drosophila melanogaster,* a majority of odorants that can be detected by the larval olfactory receptors (ORs) elicit attraction responses, with only a few acting as repellents, but the degree of attraction varies depending on the compound and concentration (Khurana and Siddiqi, 2013, Kreher et al., 2008). While there are several studies on the olfactory system and behaviour of *D. melanogaster* larvae, the knowledge of *D. suzukii* larvae is still limited.

Mating

Mate finding in *Drosophila* is usually performed by the male who locates a potential mate mainly based on visual cues (Spieth, 1974). The male then approaches the mate and performs a courtship display, typically involving movement of wings, abdomen and legs and positioning in relation to the courted individual, who will either accept or reject the courtship or not respond (ibid.).

Close range hormones are used by the flies for identification of potential mates upon contact, in *D. melanogaster* and closely related species cis-vaccenyl acetate (cVA) is used as an aggregation pheromone (Lebreton et al., 2012). In *D. suzukii*, cVA no longer functions as an attractant (Dekker et al., 2015), but the cuticular hydrocarbons produced by the females seem to play a role in signalling that the female is mature and ready to mate (Dekker et al., 2015; Revadi et al., 2015).

Drosophila suzukii and yeasts

Yeasts are known to play an important part in the diet of both the adult and larval stage of *Drosophila*, providing nutrients found in very low levels in fruit, such as proteins and lipids (Becher et al., 2012). Due to the preference of SWD to lay their eggs in ripening and ripe fruit, substrates that are usually unbalanced in their nutrient compositions, the association with microbes that provide nutrients becomes more important (Bing et al., 2018). Bellutti et al. (2018) demonstrated that female *D. suzukii* would lay significantly more eggs on cherries inoculated with *Saccharomyces cerevisiae* than on cherries that were free from microorganisms. This indicates an important role of the presence of yeast in the fruit in the female's decision to oviposit.

It is also speculated that the female transfers yeast to the fruit while ovipositing, providing the larvae with a source of the necessary nutrients and supressing growth of non-beneficial fungi (Bing et al., 2018; Rohlfs and Hoffmeister, 2005). The yeasts benefit from this association by being transferred to new substrates and an improved genetic diversity within populations due to increased outbreeding (Reuter et al., 2007).

In particular, *D. suzukii* seems to have a strong association with *Hanseniaspora uvarum*, as this yeast species is among the most attractive to the fly (Scheidler et al., 2015) and can often be found in the alimentary canal of larval and adult SWD (Hamby et al., 2012).

Drosophila suzukii can only tolerate relatively low levels of dietary ethanol compared to saprophytic species such as *D. melanogaster* (Sampson et al., 2016). This makes *H. uvarum* a suitable symbiont, as it produces low levels of ethanol compared to other yeasts commonly found in fermenting fruit (Ciani and Maccarelli, 1997).

Hanseniaspora uvarum has also been shown to have an preventative effect on post-harvest fungal infections in strawberries (Cai et al., 2015). Since certain fungal infections of the substrate in which the larvae feed can lead to increased larval mortality (Rohlfs et al., 2005), likely due to mycotoxins produced by the fungi (Trienens et al., 2010), it is possible that this yeast provides more than just nutritional benefits to SWD larvae.

Drosophila suzukii as a pest in fruit crops

Once the SWD female has laid her eggs in a fruit it may still appear undamaged for some time, until the larvae have made significant damage inside the fruit (Bolda et al., 2010). Therefore, infested berries may be accidentally included in the harvest. This leads to issues with unmarketable fruit as insect presence in the crop may lead to rejection of the entire batch of fruit (Kinjo et al., 2013, Van Timmeren and Isaacs, 2013).

In the USA *D. suzukii* infestation is usually prevented with regular pesticide treatments, but this may lead to issues with resistance (Bolda, 2011), especially if only a small variety of active ingredients are available. Growers also need to consider the potential residue levels of pesticides remaining on the harvested crop, as not to exceed maximum allowable values (Bruck et al., 2011), and the pre-harvest interval of the selected substance. The effect of insecticides on beneficial insects such as pollinators and natural predators must also be taken into account.

Due to the problems involved in regular insecticide spraying, additional alternative control methods are needed. Only a very limited number of insecticides are approved in Sweden for use in cultures vulnerable to SWD, and none are approved specifically for use against SWD (Manduric, 2018). While treatments for other insect pests may also have an effect on SWD, the active ingredients in approved insecticides (e.g. thiacloprid and pyrethrins) are not able to entirely prevent the development of eggs and larvae already present in the fruit (Cuthbertson et al., 2014). This makes the use of alternative control methods even more important. Such methods may include mass trapping, choice of early-ripening cultivars (Hampton et al., 2014) or cultivars with firm flesh and skin (Kinjo et al., 2013, Lee et al., 2011), earlier and more frequent harvest and removal of nearby non-crop hosts (Diepenbrock et al., 2016, Liburd et al., 2014) and overripe and damaged fruit.

While harvesting at an earlier ripening stage may help prevent SWD damage, the risk is not entirely eliminated, as many host plants are susceptible to oviposition before reaching a harvestable stage (Karageorgi et al., 2017, Lee et al., 2011). Mass trapping can be effective, but needs to be used with caution, as inappropriately used traps may lure the flies into the crop and increase fruit damage (Hampton et al., 2014). A good trap design is crucial, since as few as 10% of flies visiting standard cup traps may enter the trap and drown, a number that can be improved by coating the surface of the trap with insecticide (ibid.). Measures such as increasing the entrance area of the trap or using the right colour combinations can greatly increase trap efficacy (Basoalto et al., 2013). As many drosophilids, as well as other insect species, are attracted to fermentation product based lures (Burrack et al., 2015, Cha et al., 2013, Tonina et al., 2018) a highly selective lure is desirable to avoid affecting the population of non-pest species. A high selectivity also makes the lure easier to use for monitoring purposes.

History of invasion

Drosophila suzukii was first observed in mainland Japan in 1916 and by the early 1930s it had been found in several places, including parts of China and Korea (Kanzawa, 1939).

In 1980 SWD was discovered on the Hawaiian island Oahu, and it had soon spread to several other islands (Kaneshiro, 1983).

The first records of SWD in mainland USA are from 2008, when *Drosophila* larvae were found in otherwise healthy raspberries, but the adult flies collected were at first misidentified as *D. biarmipes*, a closely related species in which the male also has a wing spot (Hauser, 2011). By 2009, the fly had been correctly identified as *D. suzukii,* and spread to most areas of California, infesting crops of cherry and raspberry, and also been found in Oregon and Florida (Steck et al., 2009) as well as British Columbia, Canada (Bolda et al., 2010).

The fly was also detected in Europe around the same time, with the first few individuals being captured in Rasquera, Spain, in October 2008 (Calabria et al., 2012). By 2012 SWD had spread to all but 13 states in the USA (Burrack et al., 2012) and many parts of western Europe, excluding the Scandinavian peninsula (Cini et al., 2012). The fly reached Denmark and the southernmost parts of the region of Scania, Sweden in 2014, and was found to have spread into several new parts of Scania and a few other southern regions by 2016 (Manduric, 2017).

The range of distribution in Sweden as of 2018 is not known, but as a part of this thesis a monitoring project in collaboration with hobby growers was carried out in an effort to map the current situation. According to predictions made by dos Santos et al. (2017) the fly may potentially spread to most of Scandinavia and Finland, excluding the northernmost regions.

Invasion by *D. suzukii* can have severe economic consequences for the fruit and berry growers in that area. Goodhue et al. (2011) calculated an estimated 37% revenue loss in untreated Californian raspberry cultivation. According to Bolda et al. (2010) yield losses due to *D. suzukii* in the USA may vary from negligible up to as much as 80%, but losses around 20% were the most common. As an invasive species, the fly may also cause harm to local ecosystems. Insect infested fruit may decrease the attraction of birds who would otherwise spread the seeds of a plant (Manzur and Courtney, 1984). Therefore, a wide variety of plants whose fruit support the development of *D. suzukii* may be impaired in their ability to spread their genes (Poyet et al., 2015).

Objective

This project aims to investigate the effect the yeast *H. uvarum* has on the behaviour of *D. suzukii* through different life stages, including larval food search, female oviposition decision, and mating locations. The objective is to address the following:

The attractiveness of *H. uvarum* to *D. suzukii* larvae

Determine which compounds are necessary for the attractiveness of *H. uvarum* to *D. suzukii* larvae and if a single compound or simple blend can have a similar effect.

Oviposition

Establish the effect of *H. uvarum* presence on oviposition.

Mating

Determine where *D. suzukii* couples are most likely to mate.

Monitoring

Assess the current (as of September/October 2018) spread of *D. suzukii* in Sweden.

Material and Methods

Drosophila suzukii rearing

The colony of *D. suzukii* used was established from flies caught in San Michele all'Adige, Italy in 2011. The flies were kept in 24±2°C at a RH of 35-55% and a 12:12 h L:D cycle and reared on the Bloomington *Drosophila* Stock Center Standard Cornmeal Medium.

Yeast

The yeast used in the oviposition assay, mating observations and larval assay was *Hanseniaspora uvarum*, strain CBS 2570. The yeast was grown as a liquid culture in minimal medium (Merico et al., 2007) and incubated at 26±1.5°C in an incubator shaker (S160D Incubator and STR6 Platform Shaker, Stuart Scientific). Where a control was used it was produced by incubating minimal medium with no yeast inoculum.

Larval assay

Larvae were collected from the rearing vials 6 days after egg-laying by dissolving the diet in distilled water and transferring the larvae to pure distilled water using forceps. The larvae were then transferred to a Petri dish containing a layer of 1% agarose gel (w/v), with a thin layer of distilled water poured on the surface to decrease the number of larvae burying.

The tests were carried out in the same type of 9–cm Petri dishes containing a thin layer of 1% agarose gel as the larvae were kept on. The Petri dishes were placed on a white surface, with four black dots marking an inner circle with a diameter of 4 cm. The setup was illuminated from above by a lightbulb. The temperature in the room was 23°C±1.5°C

Filter paper discs, 10 mm in diameter, were cut out from 90 mm Filter Paper (Ahlstrom, Munktell), and two discs were placed on opposite sides along the edge of the Petri dish.

A control, consisting of 10 µl of ethanol, was applied to one of the filter paper discs. 10 µl of the compound to be tested, diluted in ethanol to a concentration of 10 ng/µl (i.e. 100 ng of compound), was applied to the other. The ethanol was allowed to evaporate for approximately 1 minute until the discs appeared dry or, if the filter paper had taken up moisture from the agar, until the ethanol halo was no longer visible. The positions of the compound and the control were alternated for the replicates to avoid positional bias. For the tests of binary blends, two compounds were mixed in equal parts to a concentration of 5 ng/µl each (i.e. 100 ng compound in total) and tested against an ethanol control using the same method as with the single compounds.

For testing attraction, a single larva was placed in the centre of the Petri dish and observed for 5 minutes. Times were recorded when the larva was:

Making contact or top contact (see below) with the control or compound disc.

Leaving the control or compound disc after contact or top contact.

Contact was defined as the larva touching one of the filter paper discs. The time was noted when the larva first made contact with the disc, and the duration of the contact was noted. The larva passing over a filter paper disc without pausing was not recorded as contact.

Top contact was defined as the larva making stops and turns on the area of lid immediately above the filter paper disc. A larva that spent at least 2:30 min on either control or treatment disc was categorized as having 'chosen' (no distinction was made between these types of contact in the results).

If the larva spent more than 2 minutes and 30 seconds in contact with one of the compounds but then left and finished in contact with the other compound it was observed for additional 5 minutes, and the compound the larva had spent the most time in contact with, was determined to be the chosen one.

To compare the attraction to the different compounds, a preference index for each compound was calculated by scoring a choice of the tested compound as 1, no choice as 0 and a choice of the control as -1 and averaging the scores.

Tests comparing a liquid *H. uvarum* culture grown for 48 hours to a minimal medium control were performed using the same method as described for the chemical compounds, with a few differences. Third instar larvae of ages between 4 and 8 days were used, and the culture and medium control were applied directly onto the agar, with no filter paper in between.

The compounds tested were chosen from the antennally active compounds present in the headspace of *H. uvarum* as listed by Scheidler et al. (2015). Compounds that were not detected by *D. melanogaster* homologues ("Olfactory coding in insects - AG Galizia - Universität Konstanz," 2018) to olfactory receptors transcribed in the larvae (*Drosophila suzukii* larval transcriptome, unpublished data) were removed from the list. Acetoin was also included as it is a major by-product of the *H. uvarum* fermentation process (Ciani and Maccarelli, 1997) and is a known attractant of *D. suzukii,* used in lure formulations in traps (Cha et al., 2012, 2014).

The chemicals used were: Acetoin CAS No. 513-86-0 (Aldrich), Ethyl acetate CAS No. 141-78-6 (Aldrich), Isoamyl acetate CAS No. 123-92-2 (Sigma-Aldrich), Isoamyl alcohol (3-methyl-butanol) CAS No. 123-51-3 (Sigma), 6-Methyl-5-heptene-2-one CAS No. 110-93-0 (Aldrich), 3-(Methylthio)-1-propanol CAS No. 505-10-2 (Aldrich), 1-Pentanol CAS No. 71-41-0 (Acros), 2-Phenylethanol CAS No. 60-12-8 (Merck), 2-Phenethyl acetate CAS No. 103-45-7 (Aldrich).

Statistical analysis was performed in R statistical software (R Core Team, 2018). For each compound or blend of compounds a χ^2-test was used to compare the number of larvae choosing the tested compound with the number of larvae choosing the ethanol control. To compare the attractiveness of the different compounds "success" (larvae choosing the tested compound) was compared to "failure" (larvae choosing ethanol or making no choice). A general linear model fitted with binomial Poisson error distribution was performed followed by a Tukey pairwise comparison.

Oviposition assay

Artificial berries

A 40-48 hr old liquid *H. uvarum* culture was used. The undiluted yeast cultures used had a concentration between $2.8*10^6$ and $30.8*10^6$ CFU/ml.

Before the oviposition test started, some of the liquid yeast culture was diluted in minimal medium to a concentration of 1 µl/ml to produce the diluted treatment. The diluted yeast was also streaked onto minimal agar plates for colony forming unit (CFU) counts. After dilution the vials containing yeast were placed on ice until the test started.

Artificial blueberries were created by mixing 29 g Fryspulver (Tørsleffs, Haugen-Gruppen) with 20 ml blue food colouring (Dr. Oetker, Oetker Group) and 20 ml distilled water. The mixture was stirred until an even consistency was reached. The mass was rolled in cling film and cut into pieces weighing around 1.5 g each. The pieces were then moulded into a smooth shape with the help of a few drops of distilled water added to the surface. Two of these artificial berries were placed opposite each other on a moist filter paper (90 mm Filter Paper, Ahlstrom, Munktell) in a Petri dish of 64 mm high and 115 mm diameter and closed with a mesh lid.

The Petri dishes were placed, three in each, into transparent plastic insect rearing boxes with the dimensions 220*305*123 mm, each containing 100 ml of water at the bottom. The rearing boxes were sealed with plastic lids to keep a high relative humidity and prevent the artificial berries from drying out. The setup was illuminated from above with a light bulb in the same 12:12 h L:D cycle as used in the rearing.

To obtain mated females, flies were collected within 24 hours post eclosion and briefly anaesthetised with carbon dioxide. Males and females were separated and placed in rearing vials containing fresh diet. When 5 days old, groups of virgin male and female flies were put together into a rearing vial containing fresh artificial diet within 10 minutes of the start of the light period for mating. Mating couples were transferred to empty rearing vials.

Artificial berries were treated by applying a 20 µl drop of the treatment or control immediately before the mated females were added. Between the replicates the different treatments were alternated between left and right to avoid positional bias.

After mating, one female was placed in each petri dish, where she was offered a choice between two different treatments or a treatment and a control for 24 hours. Eggs laid in each artificial blueberry were counted under a microscope.

A preference index for each female was calculated by dividing the difference in number of eggs laid on each artificial berry with the total number of eggs laid by that female. An average was then calculated for all females tested on each treatment pair (n=29).

Blueberries

For the oviposition assay with real blueberries (*Vaccinum corymbosum*) a similar method as for the artificial blueberries was used, with a few differences. Ripe blueberries were bought from a grocery store and rinsed in distilled water. A small wound was made in the skin near the stalk area of the blueberry to verify that berries of equal ripeness were used,. The berries were placed with the stalk area facing down and the treatment was applied on top, at the floral end. Instead of a moist filter paper under the berries the flies were provided with a moist cotton ball. No lid was placed on the rearing boxes as a high relative humidity promotes the growth of mould on the berries.

26 replicates were performed for each treatment pair with the real blueberries.

Mating observations

Virgin flies were collected within 24 hours of eclosion and briefly anaesthetised with carbon dioxide for the sorting of males and females into separate rearing vials. 5-day-old female and 4- to 5-day-old male flies were used in the mating observations. The flies were released into a 30*30*30 cm BugDorm-1 insect rearing cage where four different treatments were placed, one on each side of the cage. The treatments used were 'Inoculated Fruit' (Figure 1 a), 'Plant' (Figure 1 b), 'Clean Fruit' (Figure 1 c) and 'Yeast' (Figure 1 d). The treatments were all prepared and put into the cages right before the females were released. Ten females were released into each cage in the afternoon the day before the mating observations were to take place. The mating observations started immediately following the males being released into the cage at lights on in the morning and continued for 1 hr. The position and starting time of each copulation were noted.

The 'Clean Fruit' (Figure 1 c) and 'Inoculated Fruit' (Figure 1 a) treatments were prepared by picking ripe 'Glen Ample' raspberries and rinsing them in distilled water. The raspberries that were to be inoculated were lightly damaged with a pipette tip while the 'Clean Fruit' raspberries were left undamaged as the two fruit treatments were meant to represent fermenting or undamaged fruit. The raspberries for the 'Inoculated Fruit' treatment were inoculated with 20 µl of a 40-48-hrs old undiluted liquid culture of *H. uvarum*, the remaining raspberries were left clean.

For the 'Yeast' treatment (Figure 1 d) 100 µl of the liquid yeast culture was placed in a 30 ml cup with a mesh lid that avoided that flies could contact or feed on the yeast.

The 'Plant' treatment (Figure 1 b) consisted of root shoots from 'Glen Ample' raspberry plants. Each shoot was trimmed to leave only the 2-3 youngest leaves and placed in a 100 ml

Erlenmeyer flask that was filled with tap water and plugged with a cotton ball to prevent flies from drowning. The placement of the treatments was alternated between replicates.

A cotton ball moistened with distilled water was placed in the middle of the cage to provide drinking water for the flies (Figure 1 e).

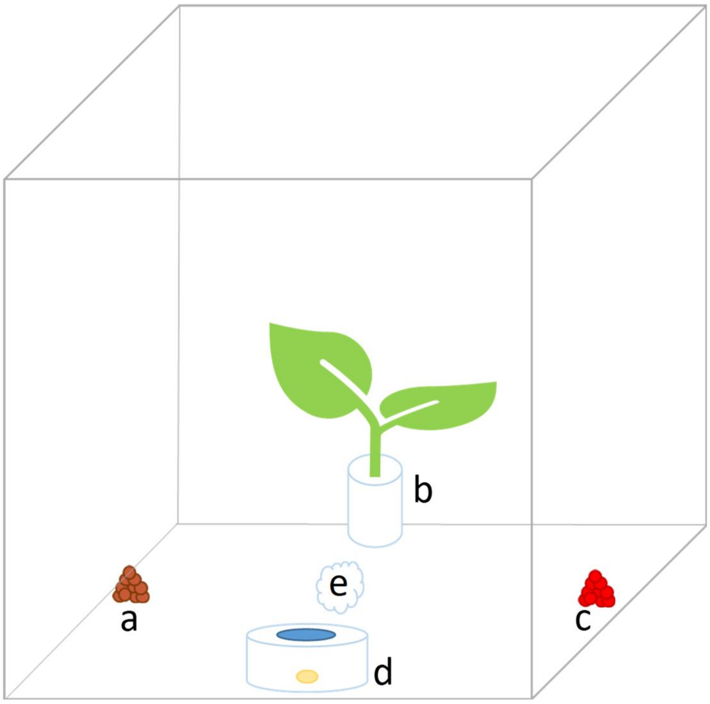

Figure 1. A sketch of the mating observation arena, containing 'Inoculated Fruit' (a), 'Plant' (b), 'Clean Fruit' (c), 'Yeast' (d) and a water source (e)

Monitoring

The monitoring was carried out in cooperation with hobby growers distributed over the different regions of Sweden. Initially, allotment associations were contacted with the help of Koloniträdgårdsförbundet, the main association organising allotment growers in Sweden. Eight allotment associations willing to participate were found this way. A further 12 locations were sampled through travelling to place traps and the help of private contacts. 2 locations were sampled by SLU forest research stations.

In total, 21 locations were sampled (Table 3). The eight allotment associations (C, E, F, G, H, I, K, T) were situated in populated areas in 6 different cities. Most of the other locations consisted of more secluded private gardens and forest locations (A, D, J, L, M, N, O, P, Q, R, S, U) Location B is a private garden but located in a relatively densely populated area.

Participators were sent a box containing 10 Riga *suzukii* traps (RIGA AG) with rain lids and wire hangers, nails with a diameter of 2.3 mm for piercing the foil lids, 10 120 ml plastic bottles for returning the trapped insects, envelopes for returning the bottles and a folder containing brief information about *D. suzukii* and the project and instructions on how to handle the traps.

The growers were instructed to hang the traps near plants bearing soft fruits or berries and leave them up for 5 to 7 days. The trapped insects were then sent back and the number of *D. suzukii* per trap was counted. The number of other drosophilids in each trap was also noted. Traps were sent out between the first and last week of September, and the last trap catches were received back during the last week of October.

Results

Larval assay

Tests where *H. uvarum* was compared to a minimal medium control resulted in 17 larvae choosing *H. uvarum* and 13 larvae making no choice, giving a preference index of 0.57, or 1.0 if only analysing larvae choosing the *H. uvarum* culture or minimal medium control, respectively.

All compounds tested were to some degree attractive to the larvae when tested against an ethanol control, but the degree of attraction to the different compounds varied greatly (Table 1). Certain compounds, mainly the acetate esters, were almost as attractive to the larvae as the *H. uvarum* liquid culture. For other compounds the larvae's preference indices were closer to 0 meaning the larvae were almost equally attracted to the ethanol control.

The larvae were also attracted to ethanol compared to minimal medium in preliminary tests, with 12 larvae choosing ethanol and 18 larvae making no choice, giving a preference index of 0.4, or 1.0 if analysis was directed to larvae making a choice of compound or control.

Table 1. The number of larvae choosing the compound, the ethanol control or making no choice, and the resulting preference indices (PI).

Single compounds (number of tested larvae)	Choice of		No choice	PI	PI (Choice only)
	Compound	Ethanol			
Phenylethyl acetate (n=30)	22	6	2	0.53	0.57
Isoamyl acetate (n=30)	17	2	11	0.50	0.73
Ethyl acetate (n=30)	16	4	10	0.40	0.60
1-Pentanol (n=30)	17	9	4	0.27	0.37
Acetoin (n=31)	14	7	10	0.24	0.28
2-Phenyl- ethanol (n=30)	13	8	9	0.17	0.33
6-Methyl-5-heptene-2-one (n=30)	14	9	7	0.17	0.27
3-Methylthio-1-propanol (n=30)	12	9	9	0.10	0.08
Isoamyl alcohol (n=30)	11	9	10	0.07	0.06

Table 2. The number of larvae choosing the blend, the ethanol control or making no choice, and the resulting preference indices (PI).

Blends (numbers of tested larvae)	Choice of		No choice	PI	PI (Choice only)
	Blend	Ethanol			
Ethyl acetate + Acetoin (n=30)	19	2	9	0.57	0.81
Phenylethyl acetate + Isoamyl acetate (n=30)	6	1	23	0.17	0.83

The blend of phenylethyl acetate and isoamyl acetate only weakly attracted the larvae and several larvae made contact with the blend and then immediately turned away. Only 7 larvae made a choice when assayed with the blend (Table 2). As a comparison, no other blend or single compound had less than 19 larvae making a choice.

The blend of ethyl acetate and acetoin was chosen because both compounds had a reasonably high attractiveness as single compounds, are present in relatively high amounts in the headspace of *H. uvarum* and belong to different chemical groups. This blend was very attractive to the larvae, which in many cases quickly contacted the blend and stayed in contact for the remaining testing time. It also had the highest preference index of all tested blends and single compounds (Figure 2).

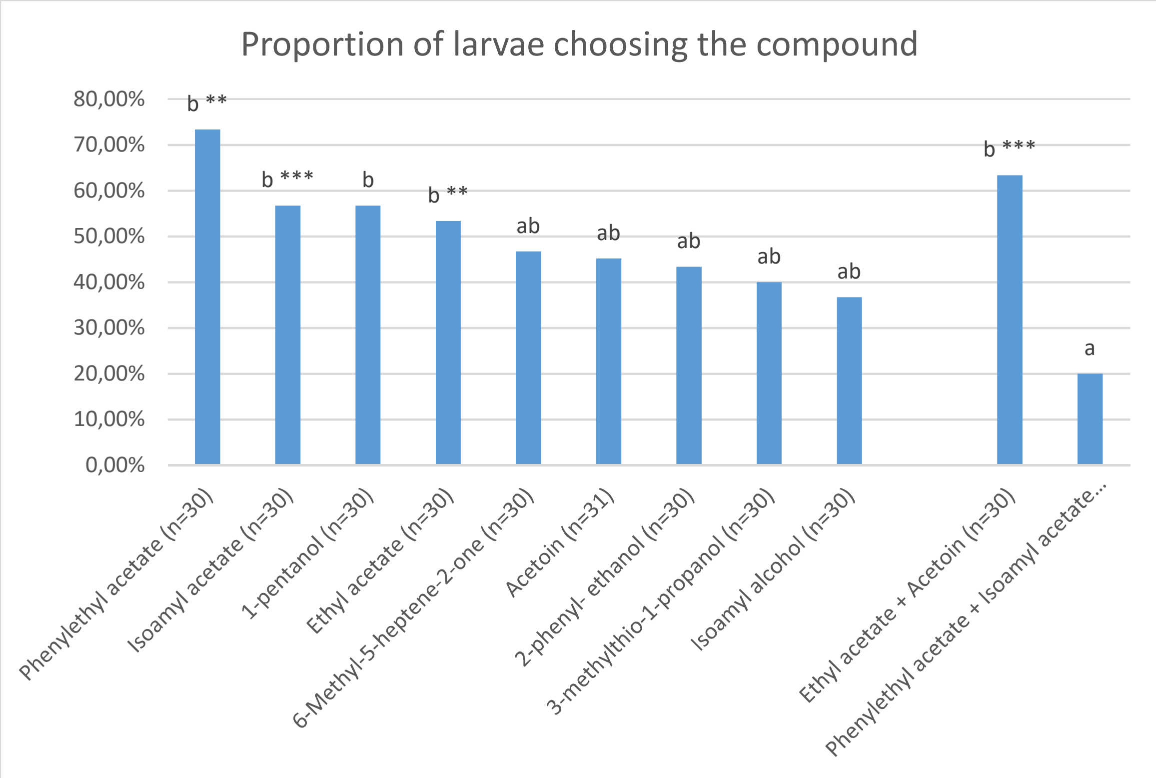

Figure 2. The proportion of larvae choosing the compound for all tested compounds and blends. Compound with significantly different attraction compared to ethanol when analysed with a χ^2-test. ** for p<0.01, *** for p<0.001. For the grouping "success" (larvae choosing the tested compound) was compared to "failure" (larvae choosing ethanol or making no choice). A general linear model fitted with binomial Poisson error distribution was performed followed by a Tukey pairwise comparison.

Oviposition assay

Artificial blueberries

Twenty-nine replicates were performed for each treatment. In the test with artificial blueberries, yeast colonies could often be found growing on the blank controls. This indicated that the flies had transferred yeast by making contact with the inoculated berries before going to the control berries.

A non-significant trend was noted toward flies with access to undiluted yeast being more likely to lay eggs (Figure 3).

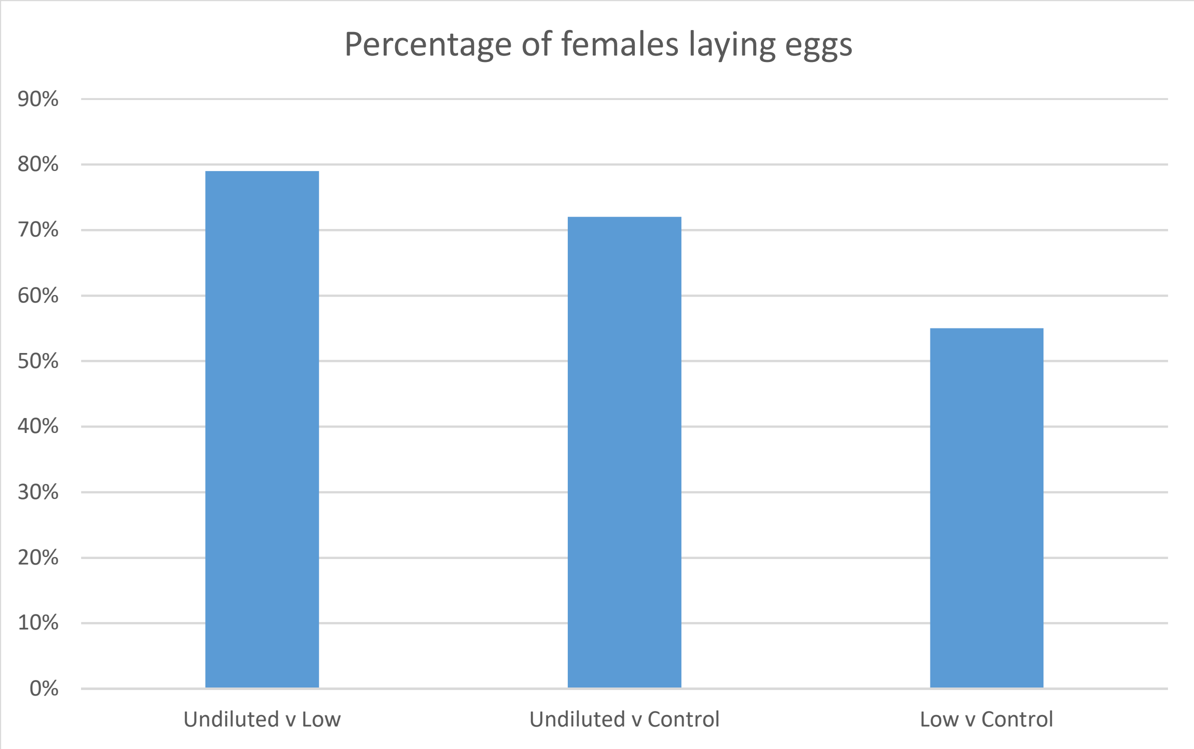

Figure 3. The percentage of tested females laying at least 1 egg in each pairing of treatments in the artificial blueberries.

No significant differences between the number of eggs laid in each treatment were found for any of the treatment pairings when analysed with Student's t-test ('Low' v 'Control' p=0.32, 'Undiluted' v 'Control' p=0.91, 'Undiluted' v 'Low' p=0.21).

Blueberries

Twenty-six replicates were made for each treatment pairing. The results were analysed with Student's t-test, but no trends in preference index or significant differences between the number of eggs laid in each treatment could be found ('Low' v 'Control' p=0.19, 'Undiluted' v 'Control' p=0.94, 'Undiluted' v 'Low' p=0.53). Despite being rinsed and checked for appropriate ripeness before being used in the assay many blueberries had started rotting or had mould growing on them by the end of the 24 hours. The average number of eggs in the mouldy berries (n=45) was 4.62, while the same number for non-mouldy berries (n=111) was 4.41. The difference was not significant.

For Undiluted vs Low and Undiluted vs Control the females laid fewer eggs in the artificial blueberries than in the real blueberries (Student's t-test, 'Undiluted v Low' p<0.01, 'Undiluted v Control' p=0.04). For Low v Control the same trend could be seen, but this difference was not significant (p=0.22).

There was also a non-significant trend toward females being more likely to lay eggs in the real blueberries when given access to undiluted yeast, with 92% laying eggs in Undiluted v Low and Undiluted v Control and 84% laying eggs in Low v Control, as compared to 79, 72 and 55% in the artificial berries.

Mating observations

It was noted that the females generally spent time resting on the walls or in the roof of the cage at the start of the light period. As the lights near the mating observation cages were delayed a couple of minutes later compared to the first lights on in the rearing room it was possible to observe the flies while still in relative darkness. Following lights on flies were observed to land on either the inoculated or the undamaged raspberries. As the males were added to the cages immediately following the lights turning on it is difficult to determine if their introduction also influenced the change in activity in the females.

A total of 190 potential couples were tested in 19 replicates, each consisting of 10 males and 10 females, resulting in a total of 96 matings, meaning 50.5% of the females and males were mating within one hour (Figure 4). No rematings were observed. A majority of the matings, 50, took place on the inoculated fruit. The second most commonly chosen treatment was the undamaged clean fruit on which 21 matings took place. The yeast and plant treatments were the least common mating sites and hosted 0 and 1 mating, respectively. The remaining 24 matings took place in places other than the treatments, typically the roof or walls of the cage.

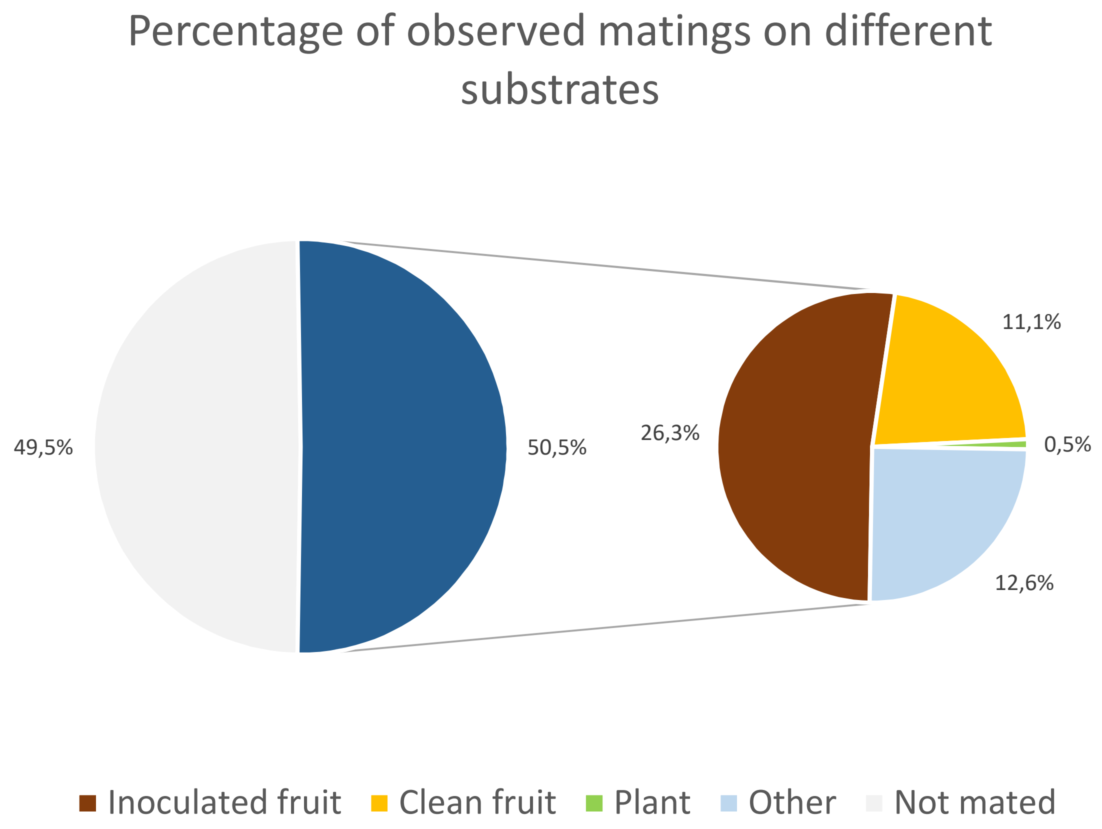

Figure 4. The distribution of matings when flies were offered a choice between four treatments

The average length of the mating on each treatment was 28.1 minutes on 'Clean fruit' (n=16), 25.9 minutes on 'Inoculated fruit' (n=23) and 24.6 minutes on 'Other' (n=12). No significant differences were found between the mating times on any of the treatments (Student's t-test, Clean-Inoculated p=0.45, Clean-Other p=0.18, Inoculated-Other p=0.53).

Preliminary tests were performed to work out the best method of carrying out the mating observations. The same method was used, but the males and females were introduced to the cage simultaneously as the lights turned on. Three to four or ten females and the same number males per cage were used, for a total of 13 replicates, 100 females and 98 males. Due to no rematings occurring this was considered as 98 potential couples. Despite flies being more likely to meet by chance due to both sexes being released into the middle of the cage simultaneously, these tests resulted in just 20 of the potential couples mating. A majority of them, 55% or 11 matings, took place on the floor or walls of the cage, where the flies came across each other soon after being introduced to the cage. Seven matings took place on the inoculated fruit in these tests, one mating took place on the clean fruit and one on the plant.

Monitoring

Drosophila suzukii was found in all three locations sampled in Stockholm and in every location south of those, with the exception of the location in Norrköping (Figure 5). No *D. suzukii* were found north of Stockholm, but locations such as Västerås and Falun had relatively high numbers of drosophilids caught (Table 3).

The highest fraction of *D. suzukii* in relation to other drosophilids outside of Scania was found in the central Stockholm (Södra Årstalunden) location (H). In this location 10.4% of the caught drosophilids were *D. suzukii.* In Alnarp, Scania the highest percentage of *suzukii* was caught, 22.3% of the total amount of drosophilids. While other caught insects were not counted, most of the catches were drosophilids. A few traps, likely ones that had been placed close to water, contained large amounts (up to roughly 50% of the insects in the trap) of mosquitoes.

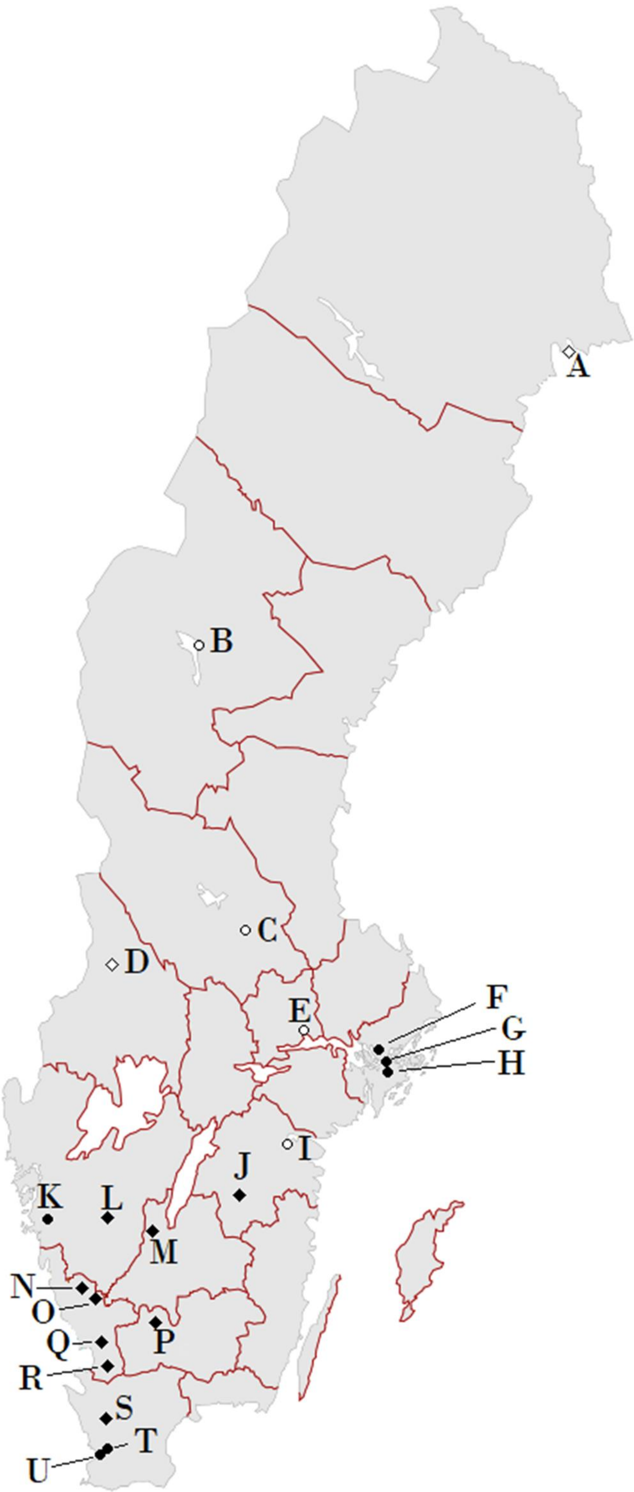

Figure 5. Map of the sampled locations. White markers indicate locations where *D. suzukii* was not captured. Black markers indicate locations where 1 or more *D. suzukii* were captured. Diamond-shaped markers indicate rural locations. Round markers indicate allotment associations and other locations in or near cities Original picture by Lokal_Profil, licensed under CC-BY-SA-2.5.

Table 3. Total number of *Drosophila suzukii* (*D.s*) and other drosophilids caught at each location

Letter	Location	Traps	Total *D.s*	Other drosophilids	*D.s*/total drosophilids
A	Luleå	8	0	118	0.0%
B	Frösön	10	0	15	0.0%
C	Falun	10	0	649	0.0%
D	Torsby	10	0	893	0.0%
E	Västerås	9	0	507	0.0%
F	Stockholm (Akalla)	10	12	282	4.1%
G	Stockholm (S. Årstalunden)	10	41	353	10.4%
H	Stockholm (Listudden)	10	2	349	0.6%
I	Norrköping	9	0	435	0.0%
J	Linköping (Ulrika)	10	1	45	2.2%
K	Göteborg (Partille)	10	4	1201	0.3%
L	Hökerum	9	27	937	2.8%
M	Elsabo	10	40	503	7.4%
N	Fagered	9	56	562	9.1%
O	Fegen	8	37	1808	2.0%
P	Lammhult	10	1	65	1.5%
Q	Halmstad	8	16	405	3.8%
R	Laholm	10	60	2150	2.7%
S	Kågeröd	10	121	3547	3.3%
T	Lund	10	9	317	2.8%
U	Alnarp	10	55	174	22.3%

Discussion

The results of the study show that yeast and fermentation products influence the behaviour of both larval and adult *D. suzukii* of both sexes. As *D. suzukii* has recently emerged as a serious pest in many parts of the world, understanding how behaviour is influenced by ecologically relevant cues and how the behaviour can be manipulated is of importance in the development of crop protection and monitoring methods. Developing efficient synthetic lures with a high attraction rate and selectivity for SWD is one such goal, and behavioural assays can help identify compounds of interest. A synthetic lure with a high larval attraction rate could potentially be used to lure larvae out of fruit as a relatively quick way of checking for SWD damage, or possibly even as an attract and kill control method. It would first be necessary to investigate if it is possible to get larvae to leave the fruit they are feeding in and navigate toward a novel odour source.

The use of minimal medium for the liquid yeast culture may have been suboptimal to reach stronger attraction, as the odour profile likely is different to that produced by yeast growing on fruit or in a richer medium. A tendency toward this could be seen when comparing trap catches using commercial bait or a liquid *H. uvarum* culture (data not shown here). *Hanseniaspora uvarum* growing in minimal medium caught very few *D. suzukii*, but the caught amount and selectivity for *D. suzukii* increased when switching the medium to potato dextrose broth. An advantage of using minimal medium is that it does not have any distinct odours that could influence the behaviour of the flies.

Larval assay

The larval assay showed that most of the tested compounds were at least somewhat attractive to the larvae. Comparably, *D. melanogaster* larvae tend to be attracted to most compounds they can detect (Khurana and Siddiqi, 2013; Kreher et al., 2008). As the compounds tested were all derived from a food source (yeast) attraction responses to varying degrees were a likely result.

Acetoin and 3-methylthio-1-propanol are yeast metabolites present in fermented products such as wine and vinegar and have been shown to increase the attraction of adult *D. suzukii* to a blend of ethanol and acetic acid (Cha et al., 2017). In the larval assay 3-methylyhio-1-propanol diluted in ethanol did not differ in larval attraction ability from the ethanol control. This could suggest a difference in which fermentation products play a role in the attraction of larval and adult *D. suzukii*. In addition, the compounds tested were all tested at the same concentration (10 ng/µl). This is not a realistic representation, as the concentration of the compounds in the yeast headspace vary, the acetate esters are present in relatively high concentrations, while compounds such as 3-methylthio-1-propanol make up a minor part (Scheidler et al., 2015).

The blend of ethyl acetate and acetoin resulting in the same preference index as the *H. uvarum* culture shows that it could be possible to create an attraction level similar to that of *H. uvarum* with a relatively simple blend of synthetic compounds. Acetoin and ethanol are known to be attractive to adult *D. suzukii* and can be used together in lures (Cha et al., 2017, 2014) . Ethyl acetate can also be used in *D. melanogaster* lures (Baker et al., 2003) and has been shown to also attract *D. suzukii* (Kleiber et al., 2014). While ethyl acetate and acetoin were not the most attractive single compounds, they are more prevalent in the headspace of *H. uvarum* than in that of several other fruit- and *Drosophila*-associated yeasts (Ciani and Maccarelli, 1997, Scheidler et al., 2015). It is possible that these two compounds together at the right ratios play an important role in allowing *D. suzukii* to distinguish *H. uvarum* from other yeasts. If a blend of just these two compounds can create a high selectivity for *D. suzukii* it could be useful in monitoring and mass trapping. Lures containing fermentation products such as wine and vinegar are attractive to a wide variety of drosophilids (Burrack et al., 2015), which leads to unwanted bycatches and complicates monitoring. This could be seen in the monitoring carried out as part of this thesis, where *D. suzukii* only made up a minor part of the drosophilids caught when using a commercial trap containing a wine-vinegar type lure.

For future experiments, mimicking the correct relative concentrations of the various compound present in *H. uvarum* headspace would improve the ability of the assay to determine which compounds are the most important for causing attraction. Giving the larvae or adult flies a choice between the most attractive synthetic blend and an *H. uvarum* culture could provide clarity about the preference for live yeast compared to a synthetic mimic.

Oviposition assay

The female choice of oviposition site is clearly a complex process influenced by many different factors. While no significant differences in the numbers of eggs laid were found between the different treatments the presence of undiluted yeast in the arena seemed to somewhat increase the female's likelihood of laying eggs. Bellutti et al. (2018) showed that *D. suzukii* females would lay more eggs on cherries inoculated with certain yeast species, including *S. cerevisiae* and *Candida* sp., and that *H. uvarum* did not increase egg laying. As the flies often carry *H. uvarum* in their alimentary canal, it is possible that the presence of this specific yeast is less important for inducing oviposition as the females may inoculate the fruit while laying eggs

(Bing et al., 2018; Rohlfs and Hoffmeister, 2005). This could be one part of the explanation for why no significant differences were found between the treatments.

Yeast colonies were often found growing on the artificial berries treated only with minimal medium (on the real blueberries yeast colonies were not visible), indicating that the flies transfer yeast between the berries. This is likely to have affected the results, and for future experiments a shorter experimental time should be considered, to limit yeast growth. Given the 24 hr experimental time, it is possible that the diluted yeast treatment had time to grow to densities similar to those of the undiluted treatment, further adding to the lack of significant differences between the treatments.

Minor differences in ripeness in the real blueberries could be another factor in the unclear results. While berries that were as similar as possible in size and ripeness to each other were used in each replicate, they could naturally not be guaranteed to be identical. As shown by Karageorgi et al. (2017), the odours associated with ripe fruit play an important role in the females decision to lay eggs, while overripe fruit is less attractive. In addition to the potential differences in ripening stages between the blueberries it is possible that the contamination by other microorganisms, such as moulds, affected the behaviour of the flies. However, it is not certain that the presence of mould would supress egg-laying (Trienens et al., 2010). No significant difference was found between mouldy and non-mouldy berries. The mould not having an effect could possibly be due to it usually being limited to the small area of the stalk scar. For future assays surface sterilizing the berries, for example through submerging them in ethanol, could lower the influence of bacteria and fungi.

The use of artificial berries could help eliminating the influence on the female's behaviour by the variation in ripeness or content of other microorganisms found in real berries. However, the tests with artificial berries resulted in a lower average number of eggs laid per female and a higher number of females not laying any eggs, when compared to experiments with real berries. As the artificial and real berries were not tested simultaneously a real comparison cannot be made due to the effect of external factors on the flies used, but it suggests there may be room for improvement in the design of the artificial berries.

The lack of fruit volatiles could be one factor that lessens the fly's egg-laying in artificial blueberries, as odours from the host play an important role in *D. suzukii*s search for oviposition sites (Karageorgi et al., 2017). The artificial berries were also softer than real berries and it is unclear if this could have affected the egg-laying. SWD females seem to prefer a certain range of substrate firmness, laying fewer eggs if the substrate is too soft (Burrack et al., 2013) unlike other *Drosophila* species who prefer softer substrates (Silva-Soares et al., 2017).

Mating observations

Based on the short-range mate finding employed by the males of many *Drosophila* species (Spieth, 1974), it could be expected that mating would be common in places where the flies gather in greater numbers, such as feeding sites. From a distance male *D. melanogaster* will navigate toward food odours and over short distances the addition of odours released by mating flies, possibly cVA, is more attractive than food odours alone (Lebreton et al., 2012). But as the function of cVA for *D. suzukii* is not the same as for *D. melanogaster* (Dekker et al., 2015), it is not clear if this mechanism is present in *D. suzukii*. Grosjean et al. (2011) showed that male *D. melanogaster* are more likely to initiate courtship in the presence of a food source sites due to a specific olfactory receptor tuned to certain fruit related odours. Similarly, Gorter et al. (2016) showed that *D. melanogaster* females were more likely to be receptive to mating in the presence of a food source, especially if the food source had a high content of yeast or sugar. In the mating observations a majority of matings took place on the raspberries, where the females were often feeding before the males were added. The raspberries inoculated with *H. uvarum* hosted more than half of the total matings which could indicate a combined effect of the females' need to feed on yeast (Mori et al., 2017; Simmons and Bradley, 1997) and the males' preference of initiating courtship near food. As red objects have been shown to be more attractive to *D. suzukii* than most other colours (Rice et al., 2016) it is possible that the colour of the raspberries also played a part in attracting the flies, increasing the encounter rate on those treatments.

In contrast, the treatment consisting of yeast odour alone, did not attract any matings. This was likely due to the fact that the flies would only spend a short period of time, if any, there before moving on, making any interaction between flies unlikely. As for the plant treatment, a few flies spent the majority of the testing time sitting passive on the undersides of the leaves and only one mating occurred there. *Drosophila suzukii*s lack of cVA (Dekker et al., 2015) may make it more dependent on visual cues for mate finding. This would make matings less common in complex environments such as plants, both due to low fly densities and obstructed visuals.

The preliminary tests where both fly sexes were introduced to the cage simultaneously and the observations starting immediately resulted in fewer matings overall. The matings that did take place often took place at the point of introduction of the females. The lower number of matings seem to at least partially have been the result of lower receptivity in the females, as the encounter rate was high, and the males did not seem to show a decrease in courtship initiation. This shows the importance of allowing the flies to remain undisturbed for a period of time in their new surroundings before starting the mating observations. It also shows that the males are less affected by the move to a new environment and will initiate courtship even if recently disturbed. To investigate if it makes a difference which sex is allowed to remain undisturbed the same experiments could be performed but introducing the males in the afternoon and the females in the morning immediately before observations start. If this results in fewer matings than when the females are introduced the day before it would further indicate that female receptivity is lowered if disturbed soon before mating.

Monitoring

In the monitoring experiment *D. suzukii* was found in all three sampled locations in Stockholm, but not in the less populated city Västerås that lies nearby. It is possible that fruit and berry trade has accelerated the spread to larger cities. It is not clear if *D. suzukii* is able to overwinter in Stockholm or if the flies caught are the result of a new introduction this year. However, finding it in three separate parts of the city (Akalla and Södra Årstalunden being around 20 km apart, with Listudden about 8 km further south) would seem to indicate that the fly is capable of surviving the winter. The fact that more than one (up to 41) *D. suzukii* was found in each Stockholm location could also suggest that the fly has been present for some time, developing populations large enough that several flies can be found when sampling a random location. The winter morph of *D. suzukii* is capable of surviving prolonged periods of time at temperatures as low as 1°C (Shearer et al., 2016). Increased access to sheltered locations, such as buildings, in populated areas may mean the fly could survive the winter further north in cities than it would normally be able to in the wild.

According to the calculations of dos Santos et al. (2017) *D. suzukii* may be able to spread as far north as the regions of Jämtland and Västernorrland, but no flies have yet been trapped there. The fact that other drosophilids were found in all sampled locations could indicate that climatic conditions would support survival of *D. suzukii* in most parts of Sweden.

As the sample sizes are small and only a limited area of each region was sampled it is not possible to exclude that *D. suzukii* is present in a certain region. No *D. suzukii* were for example caught in the sampled location in Norrköping, but it seems likely that the fly could be present there, as it was found both south of Linköping and in Stockholm. In locations such as Linköping and Lammhult only 1 *D. suzukii* was caught, showing that it would be easy to miss the presence of the fly in a location if the population was small.

As of 2018 the fly seems to be established in most of the southern parts of the country up to and including Stockholm. It can be found both in densely populated areas as well as forests and private gardens. The *D. suzukii* populations may still be low enough in most parts of the country to not cause noticeable damage. Growers of soft fruits and berries need to be aware of its presence and plan their plant protection measures and monitoring accordingly. Preventative measures such as removing overripe, damaged and fallen fruit and keeping the immediate surroundings clear of non-crop hosts are measures that should be taken even before *D. suzukii* is detected in the area, to minimise the risk of damage. Considering characteristics such as ripening time and firmness of flesh and skin when choosing cultivars would be advisable in areas at risk of future damage. This especially applies to long cultures such as blueberries and cherries where exchanging the plants from one year to another would not be economically viable, making a choice of SWD-resistant cultivars relevant even if the fly is not yet a problem in the area. Monitoring by placing traps around the perimeter of the crop should be regularly carried out in areas where *D. suzukii* has been found but is not yet causing crop damage. This would enable growers to take plant protection measures in time, avoiding *D. suzukii* population growth that could result in major economic damage.

Conclusions

The strong attraction of larvae to the blend of acetoin and ethyl acetate shows that attraction similar to that caused by *H. uvarum* can be created with a relatively simple blend of synthetic compounds.

Oviposition seems to be positively influenced by the presence of undiluted yeast, but no significant differences could be seen between different treatments, possibly due to the transfer and growth of yeast during the relatively long run time of the assay.

Mating seems to primarily take place on fermented fruits, where the females, and to a lesser extent the males, are feeding on yeast. This is likely due to the combination of the females need to spend time feeding on yeast and the males increased courting when near a food source.

The monitoring shows that *D. suzukii* is now present in most parts of the country up to and including Stockholm. This means growers outside Scania, who have previously not been affected, now need to take this new pest into account when planning their plant protection measures. Regular monitoring should be carried out in most of the country to track the arrival and population development of *D. suzukii*.

Acknowledgements

I would like to thank everyone involved in the making of this thesis. I especially thank my supervisor and co-supervisor, Paul Becher and Guillermo Rehermann, for all their help both in the design and carrying out of the experiments and their input on the writing of this manuscript. Thanks to Santosh Revadi for introducing me to the chemical ecology group and helping with the initial planning of the thesis structure.

I also want to thank everyone in the chemical ecology horticulture group for their help discussing the results along the way and providing ideas for improving the design of the various experiments.

Everyone else who has been involved in the SWD group during my time working on my thesis deserve thanks for their help with the fly rearing as well as answering my questions and providing pleasant company in the lab.

Thanks to SLU-LTV faculty´s Martha och Fredrik Nilssons Fond and Gösta och Anna-Birgit Henrikssons fond for providing funding for the monitoring project and to BioBasiq Sverige AB for offering a discount on the Riga traps.

Finally, a thanks to all participators in the monitoring project for taking the time to help with the insect trapping.

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