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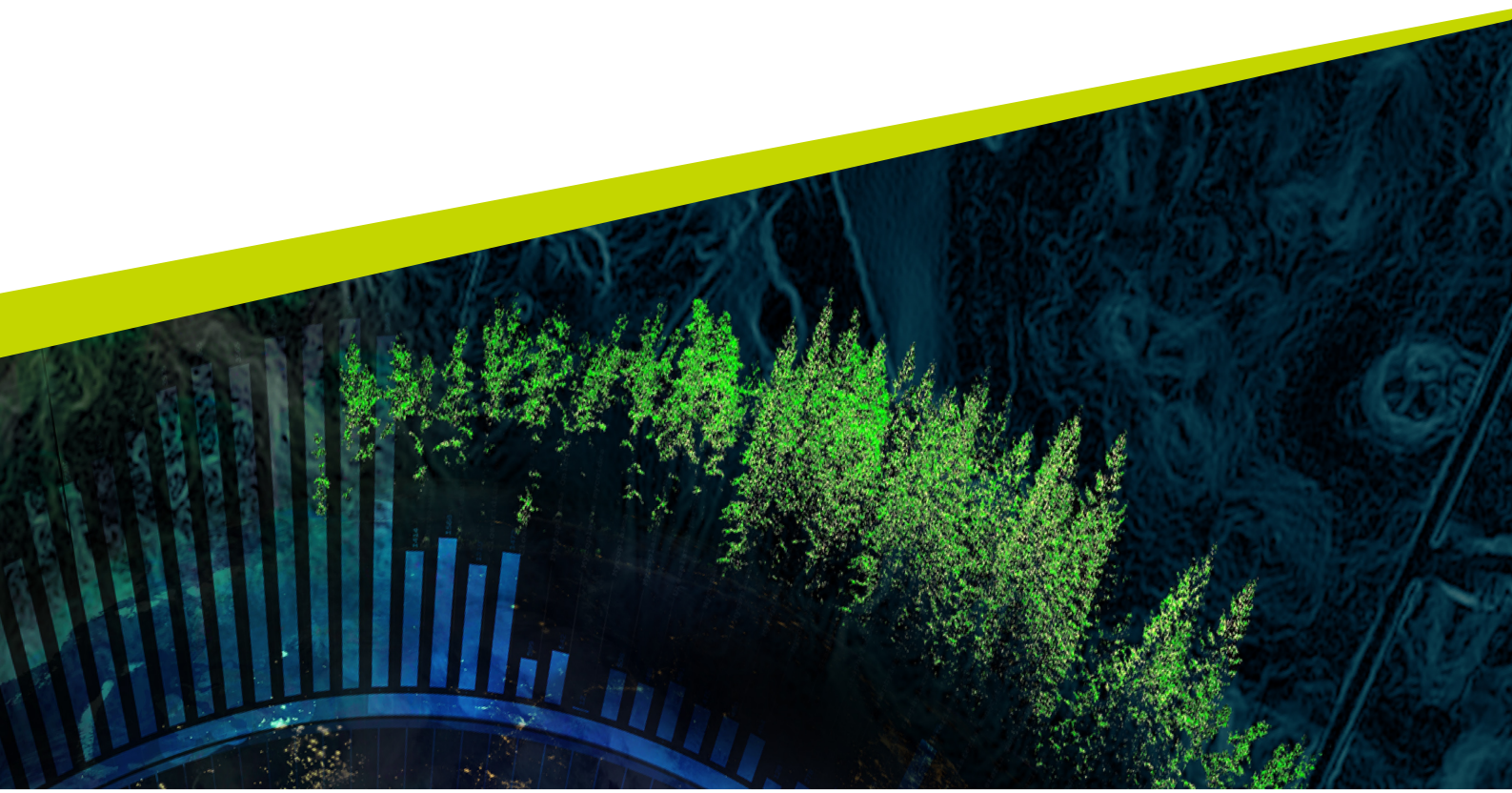
Karolina Sahle

The role of fruit and yeast volatiles in mating and oviposition of the invasive fly *Drosophila suzukii*

Betydelsen av doft från frukt och jäst för parning och äggläggning hos den invasiva flugan *Drosophila suzukii*

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Abstract

Drosophila suzukii is a serious pest of soft skin fruit and berries. Despite *D. suzukii* niche to lay eggs in ripening fruit, they seem to have a similar association with yeast as other drosophilids. Yeast provides larvae and adult flies with essential nutrients and the flies contribute to yeast dispersal when moving between plants. Like other drosophilds *D. suzukii* is believed to have an association with a specific yeast flora where *Hanseniaspora uvarum* is known to be the predominant species. The object of this thesis has been to investigate the impact of microbial volatiles from the associated yeast *H. uvarum* in mating and oviposition choices for *D. suzukii.*

In a no-choice assay, individual couples of virgin *D. suzukii* flies exposed to volatiles from *H. uvarum* were more likely to mate during the 30-minute experiment compared to the blank control. In a second experiment to assay the effect of the minimal growth media and higher relative humidity in the *H. uvarum* treatment we got a weak response and no difference between treatments and control suggesting that further experiments should be conducted for solid results. Volatiles from *H. uvarum* or raspberries, or a combination of these, was found to evoke oviposition to an equally high extent in mated *D. suzukii* in absence of visual cues compared to the control. The results indicate that *D. suzukii* uses volatile microbial cues when deciding to mate and oviposit.

Keywords: microbial volatiles, reproduction, yeast, fruit, raspberries

Popular scientific summary

Invasive pest fly uses the smell of yeast when making important life choices

Raspberries, blueberries, and cherries are all nutritious and delicious. Unfortunately, not only to humans. The spotted wing drosophila (SWD), *Drosophila suzukii*, a fly native to Japan, has recently been spreading to Europe, America, and Africa where it presents a serious threat to the cultivation of soft fruit and berries. Opposite to the closely related vinegar fly, who you might find when leaving fruit unattended in the kitchen for too long, SWD lays eggs in ripe and ripening fruit in the field which leads to destroyed fruits and spoiled harvest.

In our experiments, we looked at a yeast known to be especially attractive to SWD, *Hanseniaspora uvarum*. We found that flies who did smell this yeast to be more willing to mate and to lay more eggs compared to flies not exposed to smelling yeast. Our experiments show that the smell from yeast living on the fruit is used by the fly to make choices for where to mate and lay eggs. These choices are also interesting to us, fruit and berry eaters, who might prefer a berry free from larvae.

Knowledge about what microorganisms that are especially attractive to SWD can be used in pest control, to attract the flies to a trap baited with yeast or to a mix with insecticides. Hopefully, this could contribute to a more specific pest management of SWD flies, and less non-target killing of beneficial insects.

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1. Introduction

This thesis has aimed to look further into the relationship between the invasive pest fruit fly *Drosophila suzukii* and the associated yeast *Hanseniaspora uvarum*. The goal was to examine if odours emitted from *H. uvarum* were sufficient to stimulate mating and oviposition in *D. suzukii.* Research on this subject could contribute to further understanding of the tri-trophic interactions between insects, plants, and microbes. Knowledge about how the volatiles from microbes are affecting insect reproductive behaviour could be used in the development of plant protection strategies.

1.1. Insect and microbe mutualisms

Insects are a huge group of organisms that inhabit very diverse niches, partly enabled by symbiosis with microorganisms (Janson et al. 2008). All insects are colonized by microbes and most cells in a healthy organism are microbial (Douglas 2015). Symbiotic microbes are found in different parts of the insects, especially on the exoskeleton, in the gut, or within insect cells (Douglas 2015). Sometimes the insect and the microbes both benefit from the association, such association is called mutualism (Bronstein et al. 2006). Insect microbe mutualisms for example enable insects to feed on less nutritious food sources where the microbes provide the insect with nutrients, they cannot synthesize by themselves, for example; essential amino acids, sterols, and vitamin B (Lauzon 2003; Douglas 2015). For insects feeding on wood, microorganisms can assist with the degradation of the wood and be crucial for the insect lifecycle (Calderón-Cortés et al. 2012; Douglas 2015). Microbes can protect insects against pathogens, detoxify metabolites, and produce social cues (Lu et al. 2016). The microbes benefit through vectoring,

dispersal, and protection by the insect (Gilbert 1980; Ollerton 2006; Buser et al. 2014).

Microbes emit volatiles and are involved in microbe-insect interactions. When a substrate is colonized with microbes the odour profile may change and might be used by the insect for information about the nutritional value of the substrate (Tasin et al. 2011). Microbial volatile organic compounds (MVOCs) serve as chemical cues or signals that provide the insects with information like the nutritional quality of a food source, mating opportunities, or where to find suitable oviposition sites (Davis et al. 2013).

1.2. Drosophilids and yeast

Drosophilids are known to be associated with yeast which provide food to adult and larval flies (Starmer & Fogleman 1986; Hamby et al. 2012). The relationship between yeast and drosophilids generally is considered mutualistic where the fly benefits from the association through increased nutritional value or detoxification of the food (Stamer 1986; Anagnostou et al. 2010). Yeast diet has been found to increase the magnitude of eggs laid by *Drosophila* females (Simmons 1997; Chippindale 2004). Oviposition substrates colonized by yeast are often preferred oviposition sites for drosophilids compared to oviposition sites that are affected by bacteria or moulds (Hamby 2012; Oakeshott 1989). Yeasts benefit from symbiosis through vectoring and spore dispersal when the fly moves between different host plants and when transferred between the flies during courtship (Starmer et al. 1988; Ganter 2006; Reuter et al. 2007). Even though diverse yeast communities and a mixture of different yeasts seem to be a more attractive food source than a single yeast species (Starmer & Fogleman. 1986), there is a species-specific yeast preference in the *Drosophila* larval diet which probably is important for the species separation and occupation of different niches in a shared environment (Starmer & Phaff 1983; Becher et al. 2012). A study by Becher et al

(2012) about the model organism *Drosophila melanogaster* and the yeast *Saccharomyces cerevisiae*, showed that yeast alone induced fly attraction and oviposition, and yeast odour was a stronger attractant compared to fruit odour. Females of *D. melanogaster* laid more eggs and produced more offspring on grapes inoculated with yeast.

1.3. *Drosophila suzukii*

Drosophila suzukii (Matsumura) (Diptera: Drosophilidae) is a pest of soft skin fruits and berries (Walsh et al. 2011; Lee et al. 2011; Hauser 2011). The common name spotted wing drosophila (SWD) refers to the characteristic of the dark spots on the wings of the male flies. *Drosophila suzukii* originates from Asia and was first reported in Japan in 1916 (Kanzawa 1939). *D. suzukii* is invasive in America and Europe where it was first reported in 2008 and where it has been spreading rapidly since (Calabria et al. 2012; Asplen et al. 2015). Findings of *D. suzukii* have recently been reported from the African continent (Boughdad et al 2020; Kwadha et al. 2021).

Female *D. suzukii* has an enlarged, serrated ovipositor which allows them to penetrate and lay eggs in ripening fruits and berries, which causes serious economic loss (Bolda et al. 2010; Walsh et al. 2011). The fruit skin injury enables secondary microbial infections and allows other *Drosophila* species to infest the fruit which leads to rapid decay (Walsh et al. 2011; Cini et al. 2012; Ioriatti et al. 2015). A female *D. suzukii* lays up to 600 eggs during their lifetime which in combination with fast lifecycles with up to 13 generations a year facilitates a very fast spreading (Kanzawa 1939; Tochen et al. 2014). High relative humidity has a positive impact on the fly life length and number of laid eggs (Tochen et al 2016a).

Drosophila suzukii prefers red or black coloured oviposition sites, which corresponds to their hosts in their native environment like autumn olive

(*Elaeagnus umbellate* Thund.) and buckthorn (*Frangula* spp) (Kirkpatrick et al. 2015; Lee et al. 2015; Rice et al. 2016). In a study on different species and stages of maturity in high bush blueberries, *D. suzukii* were more attracted to berries that had higher contrast between leaves and berries (Little et al., 2018). Entling et al. (2018) showed that the resistance of the skin of the fruit, as well as prior damage, were important factors for *D. suzukii* oviposition in grapes. In prior experiments, *D. suzukii* has been shown to oviposit in agar when odour from strawberries was present (Karageorgi et al. 2017).

Drosophila suzukii has a large oviposition host range, where raspberries represent one of the preferred fruits (Bellamy et al. 2013). Current pest management strategies often are based on broad-spectrum insecticide applications (Beers et al. 2011; Bruck et al. 2011; Van Timmeren & Isaacs. 2013). The use of pesticides is one of the major reasons for insect biodiversity loss (Gibbs et al. 2009; Geiger et al. 2010; Rundlöf et al. 2012; Goulson 2013). Hence finding ways for more specific and less harmful ways for plant protection and human health is urgent.

1.4. Association of *Drosophila suzukii* and *Hanseniaspora uvarum*

Drosophilids often have specific yeasts as symbionts, and despite *D. suzukii* niche to oviposit in ripening fruits, they are believed to have a similar association to yeast as other drosophilids that typically oviposit on overripe fruit (Iglesias et al. 2014; Hamby & Becher 2016). Hamby et al. (2012) examined the different yeast species found in *D. suzukii* midguts, frass, and fruit hosts and found *H. uvarum* to be the predominant one. Furthermore, mated *D. suzukii* females have shown an increased attraction to *H. uvarum* volatiles and fed more *H. uvarum* compared to virgin flies (Mori et al. 2017). Bellutti et al. 2018 found that *D. suzukii* larval development was positively affected by a diet containing *H. uvarum* or *Candida* ssp. They also found that females fed with *Candida* ssp. laid more eggs compared to flies not fed with yeast. In a laboratory assay, *D. suzukii* was found to be equally

attracted to *H. uvarum* sprayed on grape leaves compared to ripe grapes (*Vitis vinifera*) (Rehermann et al. in prep). *D. suzukii* attraction to *H. uvarum* volatiles has recently been shown by Castellan et al (in prep). Overall, these results suggest a strong association between *D. suzukii* and *H. uvarum*

Hanseniaspora uvarum is a commonly occurring yeast in grapes and other fruits (Langenberg et al. 2017) and is found to be one of the predominant yeast species in the early stages of fermentation in grapes while disappearing in favour for other species at a later stage (Ciani et al. 2006; Hong & Park 2013). *H. uvarum* has further been suggested to be a useful agent in the biocontrol of moulds such as *Botrytis cinerea* (Liu et al. 2010).

1.5. Reproductive behaviour and food odours

Grosjean et al. (2011) found that fruit odours from phenylacetic acid induce male courtship behaviour in the model organism *D. melanogaster* via the ionotropic receptor IR84a. Gorter et al. (2016) found that female *D. melanogaster* mated more when exposed to yeast sensed by ionotropic receptor Ir8a. In the experiment from Gorter et al. (2016) the couples exposed to yeast mated more times during a 24-hour period compared to control, but the presence of yeast did not affect the time of the first mating in virgin flies.

The presence of yeast *(Saccharomyces cerevisiae)* in the oviposition substrate increases oviposition in *D. melanogaster* (Becher et al. 2012). Previous research on *D. suzukii* shows that the flies are attracted to the volatiles from fresh undamaged fruit (Revadi et al. 2015a) and that the volatiles from fresh fruit induces oviposition (Karageorgi et al. 2017).

1.6. Objectives and hypothesis

This project has aimed to further investigate the association between the pest insect *D. suzukii* and their associated yeast *H. uvarum*. The importance of microbial volatiles for mating and oviposition in *D. suzukii* is not yet clearly understood. This study hypothesized that odours, emitted by *H. uvarum* stimulate mating and oviposition behaviour in *D. suzukii*.

Research questions

1. Are odours from *H. uvarum* sufficient to stimulate mating in virgin *D. suzukii?*

2. Are odours from the yeast *H. uvarum*, fresh raspberries or a combination of the two sufficient to induce oviposition in *D. suzukii* on an agar plate?

2. Materials and methods

Drosophila suzukii from a wild type strain collected at Hallongården, (Trelleborg, Skåne, Sweden) were reared in the laboratory for about 80 generations at a temperature of 23 ± 2 °C and relative humidity above 40 %. The photoperiod was 12L: 12D (Light: Dark) with the light period between 8:30 am to 8:30 pm. The flies were kept in plastic vials filled with about 2 cm of *Drosophila*-diet (water 6330 ml, cornmeal 462 g, plant agar 24 g, malt 132 g, yeast 109,5 g, soymeal 63,24 g, sugar syrup 486 ml, propanoic acid 30 ml). The newly emerged flies were daily collected, sorted by sex during shortly CO_2 anesthetization, and placed in separate vials in groups of about 20 flies.

For all bioassays, *H. uvarum* (CBS2570) liquid cultures were freshly grown. An inoculum of 100 µl of *H. uvarum* was placed in about 50 ml of PDB (Potato dextrose broth; Difco) and grown for 24 hours to reach an optical density (OD) of approximately 0.150 ± 50, measured at 595 nm using a UV-1800 UV/Visible Scanning Spectrophotometer; 115 VAC (Shimadzu).

2.1. Mating assay

In this experiment, we tested if the mating activity in *D. suzukii* was affected when flies were exposed to the odour from *H. uvarum.* For the experiment 4-day old virgin flies were used. The experiment was conducted between 9 am- 10 am according to a study of the sexual behaviour of *D. suzukii* (Revadi *et al.* 2015b) which showed that *D. suzukii* preferred to mate during the first 3 hrs of the photo phase. The flies were assayed for 30 min and thereafter removed and new couples were placed for a new test.

The arena (see Figure 1), was built from one glass dish, 500-ml volume, height 65 mm, diameter 115 mm (VWR, 2016-0073). The glass dish was covered with a fine mesh (Sintab 0,250 mm). In the treatment, a small glass Petri dish (10 x 50 mm) was placed inside the big glass dish containing 5 ml of *H. uvarum* liquid culture. On the mesh, four plastic Petri dishes 10 x 35 mm (Falcon) were placed. Under each of the four Petri Dishes individual couples of flies were placed and sexual behavior was observed for 30 min. First, all the males were placed and thereafter the females. The whole experiment was covered by a transparent plastic box (30 x 22 x 12 cm) to prevent odour contamination between the treatments. We observed the flies every minute and took notes about what minute a couple started to mate. The flies were used only once for the experiment and thereafter removed from the colony. Statistical analysis using Fisher's exact test was made to compare how many couples mated in each treatment.

H. uvarum with blank control
For the first experiment, we tested 5 ml of *H. uvarum* compared to a blank control.

H. uvarum with PDB and water control
To investigate for a possible effect of relative humidity and the liquid growth medium we replaced the blank control with two other control treatments, one with 5 ml of PDB and another with 5 ml of water. Higher relative humidity has been shown to affect reproduction in *D. suzukii* positively (Tochen et al 2016a).
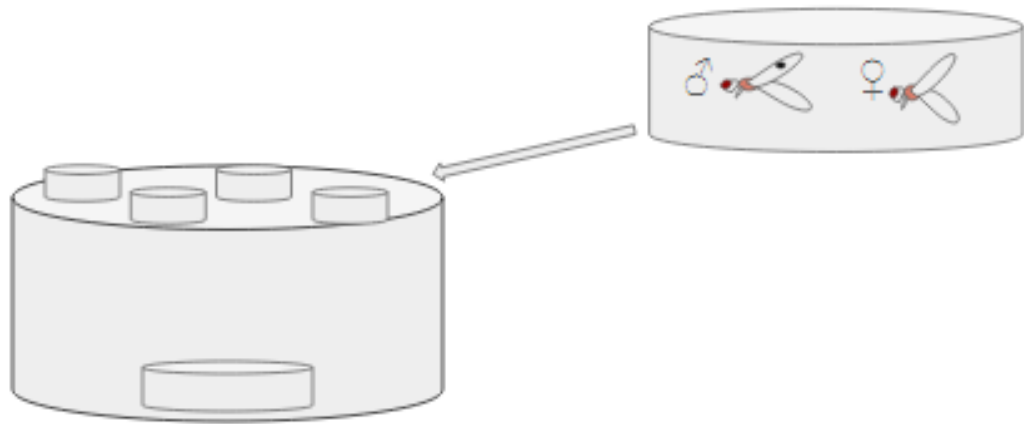
Figure 1. Experimental design of the mating experiment. The treatment was placed on the bottom of the glass dish, the dish was covered with mesh and the individual couples of flies were placed on the mesh under small plastic Petri dishes. The whole experiment was covered with a large plastic box to avoid contamination between the treatments (not in the figure).

2.2. Oviposition assay

In this experiment, we tested how oviposition was affected by volatile cues from raspberries (*Rubus idaeus*) and *H. uvarum*. The experiment was conducted in darkness, covered by cardboard boxes and black plastic bags to prevent visual cues. We used agar plates (0,5 % agarose) as oviposition substrates. Before the experiment 4-5-day old virgin females and males were brought together and when observing a mating the couple was placed in a separate vial. The mated females were thereafter used in the experiment about 1 hr later. The experiment started at midday and the flies were kept in the arena for 24 hours. The eggs laid in the agar plates were counted under a microscope.

The arenas were built by two glass dishes, 500-ml, height 65 mm, diameter 115 mm (VWR, 2016-0073) separated with a metal mech (see Figure 2). The female fly was placed in the lower part, together with the agar plate and a humified piece of cotton. The treatments were put in glass Petri dishes and placed on top of the metal mesh and covered with the second glass dish. Directly after placing the flies,

the separate treatments were covered. Before each experiment, the glass containers were washed with perfume-free dish soap and then heated in an oven at 375 degrees for 8 hours to remove possible odour contamination. Each test contained all different types of treatments.

Agar

100 ml agar medium was prepared by 4 ml glucose (D-(+) glucose, Sigma), 0,5 ml agarose (Sigma) and 0,5 ml red colorant, and 95 ml water (Dr. Oetker). The agar concentration was 0,5 % in the final product. Colorant was added to simplify the egg counting. The agar was heated in a microwave until the solvent was fully mixed, left to cool, and thereafter poured in plastic plates, 4 cm in diameter and 5 mm high with holes in the bottom (about 12- 18 mm) covered with plastic film (parafilm "M", Neenah).

Fertility assessment

After the experiment all flies were immediately placed in a fertility assessment. Each female was put in separately labelled vials with one blueberry (*Vaccinium corymbosum*) bought in local food stores. The vials were closed with a cotton ball soaked in some water to keep the flies hydrated. The females were kept in the vials for 24 hrs thereafter eggs laid in the blueberries were counted under a microscope and the result were statistically analysed.

Treatments

The treatments tested were:

Raspberries (R): Five fresh, undamaged raspberries. Conventional Raspberries (*Rubus idaeus)* were bought at the local food stores and rinsed with distilled water. New raspberries were bought for each day of testing. The raspberries were without any visible mould or damage when starting the experiment

Hanseniaspora uvarum (Y):5 ml of *H. uvarum*, preparation as explained above.

Raspberries plus yeast (R+Y): Five fresh undamaged raspberries dipped in *H. uvarum* culture.

PDB (Control) 5 ml of Potato dextrose broth (PDB, Difco). Prepared as the instruction on the package with 24 g of powder for 1 litre purified water. The PDB was thereafter autoclaved, 125 degrees for 20 minutes, and kept in the refrigerator until the experiment started and between the replicates.
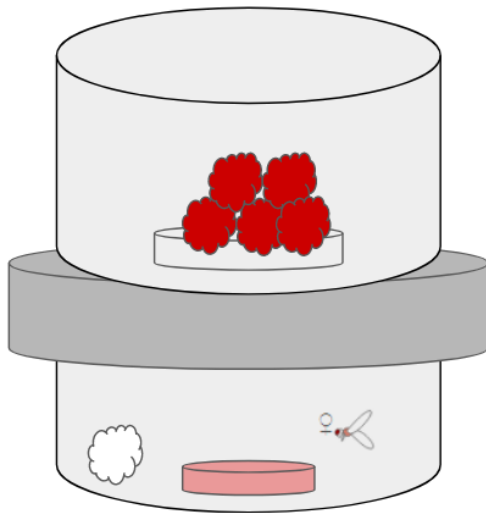

Figure 2. Arenas for oviposition experiment. The arenas were built by two glass dishes, 500-ml, height 65 mm, diameter 115 mm (VWR, 2016-0073) separated with a metal mesh. The female fly was placed in the lower part, together with the agar plate and a humified cotton ball. The treatments were put in glass Petri dishes and placed on top of the metal mesh and covered with the second glass dish. The illustration shows five raspberries as one of the treatments.

Figure 3. Pictures of the experimental setup for the oviposition experiments. All treatments were tested at the same time and kept under cardboard boxes to exclude visual cues.

In total 133 flies were tested, the flies that lay eggs neither in the treatment nor in the fertility assessment were removed from the data, leaving n=94 for further data analysis. The number of eggs in treatment was statistically analysed both for the number of eggs laid by each female and for how many of the females laid one or more eggs compared to the ones not laying any eggs. The number of eggs laid by each female in the fertility assessment was analysed as well as the average number of eggs laid by each female combining the treatment and the fertility assessment.

2.3. Statistics

For statistical analysis of the mating experiment, we compared how many flies mated when exposed to *H. uvarum volatiles* compared to the control with Fisher's exact test. The percentage of flies that mated in each treatment was calculated in excel. Fisher's exact test was also used for analysis of the second mating experiment for comparison between how many flies mated in *H. uvarum,* PDB, or water treatments, as we found no significant difference no correction method was used.

For the oviposition experiment, the number of eggs laid in each treatment in the oviposition assay was analysed by a One-way ANOVA followed by Tukey's pairwise comparisons. To compare the number of flies that laid one or more eggs in the experiment compared to the flies that laid no eggs the percentage was calculated in excel, Fisher's exact test was used for statistical analysis. Since Fisher's test can give false significance when used for multiple comparisons, and we tested four treatments, the Bonferroni method was used for correction. All statistics were conducted in Minitab19 or Excel.

3. Results

3.1. Mating assay

H. uvarum with blank control

The flies exposed to volatiles from 5 ml of *H. uvarum* did mate to a higher extent compared to the flies in the blank control (P<0,05, Fisher's Exact test). In total 88 couples were tested (*H. uvarum n= 48* and blank control n= 40). In total, 52 (i.e. 59 % of the 88 couples) of the tested couples mated within the 30 min test period of which 37 (77 %) of the couples mated in the *H. uvarum* treatment compared to 15 (38 %) couples in the blank treatment (Figure 4). In the *H. uvarum* treatment, flies were observed mating from 2 to 28 min after the start of the experiment with most flies (21 couples) mating within the first 15 minutes of the assay and 16 couples in the later part (Figure 5). In the control, the flies were observed starting to mate between 2 and 25 min from the experimental start with 13 out of the total 15 matings within the first 15 minutes (Figure 6).
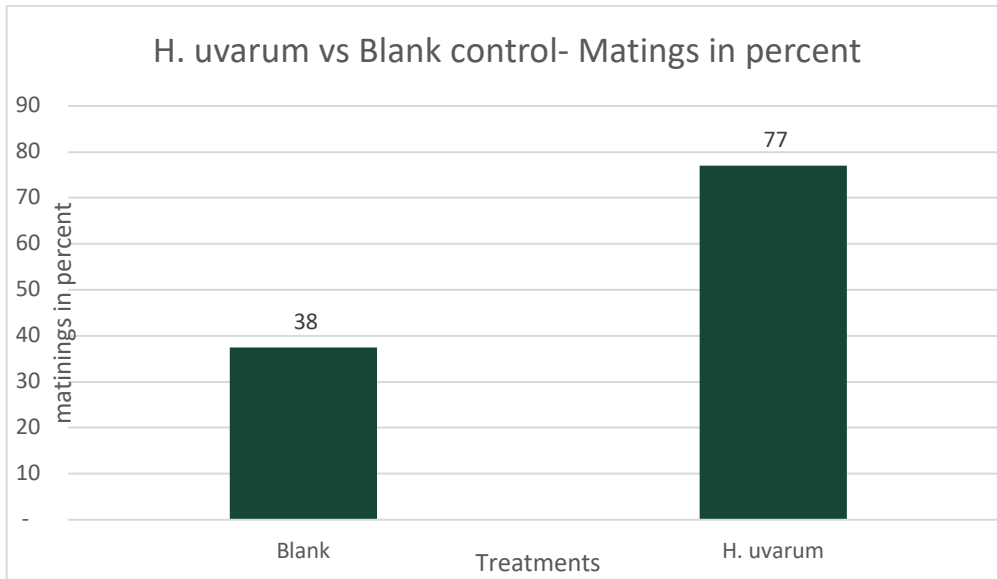
Figure 4. Percent of couples exposed to volatiles from yeast (n=48) or blank control (n=40) that mated during the 30 min experimental time.
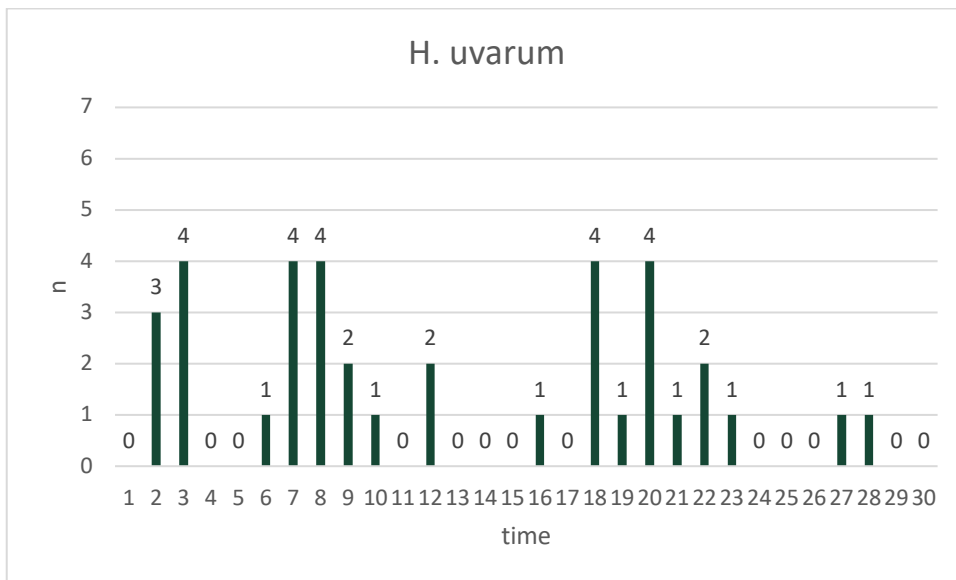

Figure 5. Number of couples initiating mating during progression of the 30 min test period when exposed to volatiles from H. uvarum (n= 48).
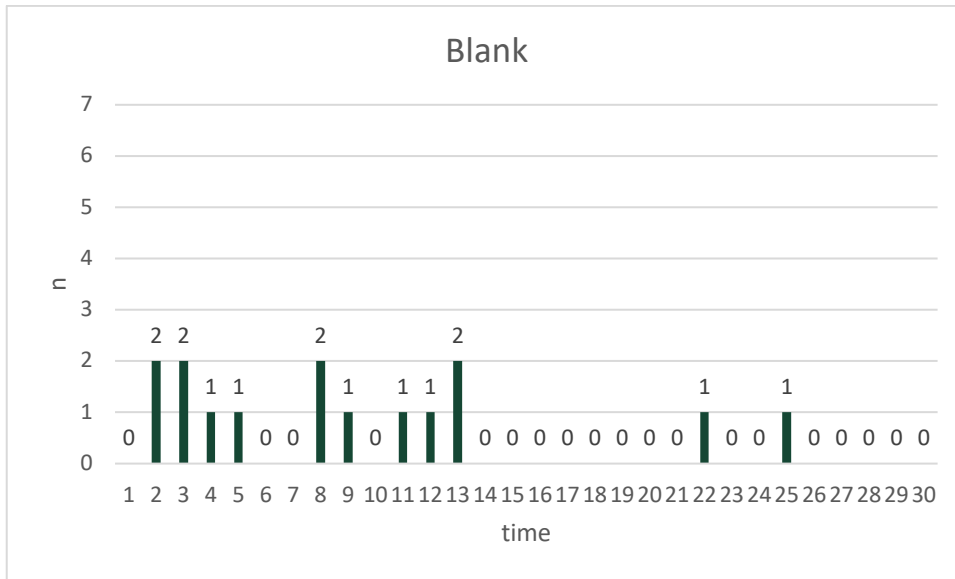
Figure 6. Number of couples initiating mating during progression of the 30 min test period in the blank control (n= 40).

H. uvarum with PDB and water controls

We found no significant difference between the yeast and the controls P>0,5 (Fisher's exact test). However, of the in total 62 couples that were tested (*H. uvarum* n= 22, water n= 20 and PDB n= 20) only 24 (39 %) showed mating. In the PDB control 7 couples mated (35 %), 8 couples (40%) mated in water control and 9 in the *H. uvarum* treatment (40,9 %) (see Figure 7).
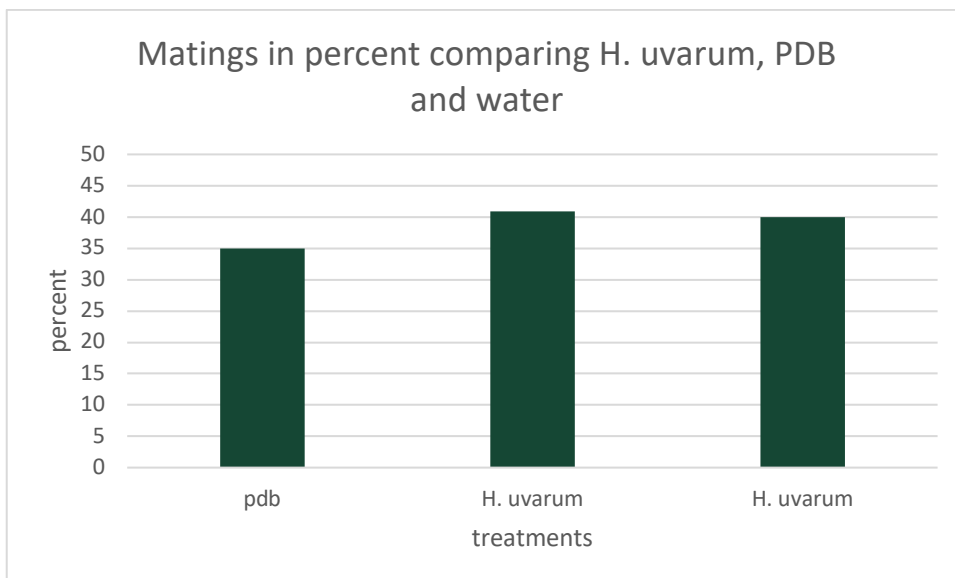

Figure 7. Percentage of couples that were mating when exposed to volatiles from PDB (n= 20, total number of matings = 7), yeast (n= 22, total number of matings = 9), or water (n=20, total number of matings = 8) during the 30 min experimental time.

3.2. Oviposition assay

In the assay to test if female oviposition is affected by volatiles from raspberry (R), raspberry and *H. uvarum* (R+Y) or *H. uvarum* (Y) compared to the PDB control we found that the flies in the control were less likely to oviposit ($P<0,05$, Fisher's exact test) and laid fewer eggs compared to all the treatments (One way-ANOVA, Tukey's pairwise comparisons). Among the treatments, we found no significant difference. The number of flies tested, and their response is shown in Table 1.

Table 1. Number of flies that were tested, flies that did oviposit and the number of eggs found in each treatment.

Oviposition experiment	Raspberries	*H. uvarum*	Raspberries plus *H. uvarum*	PDB	Total
n total	33	33	33	34	133
n valid	23	26	23	22	94
n with eggs found in treatment	20	22	19	3	64
Number of eggs found in treatment	120	137	153	14	424
n with eggs in fertility assessment	10	17	13	20	60
Eggs in fertility assessment	38	81	46	68	233
Eggs in fertility assessment and treatment combined	158	218	199	82	657

Oviposition experiment

The flies in the PDB control laid in total 14 eggs during the test phase of 24 hrs, which was a significant lower amount of eggs compared to all of the treatments (F = 8, 34, d.f. = 3, P < 0,05; One-way ANOVA, Tukey's pairwise comparisons). Between the different treatments we found no significant difference (see table 2; Figure 8).
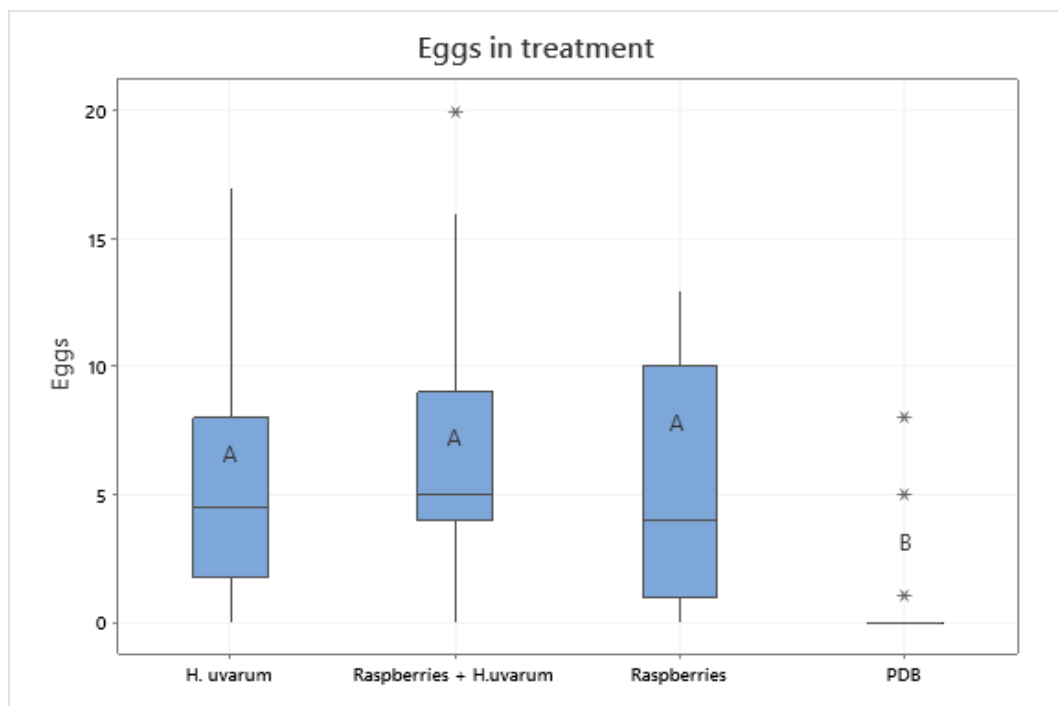

Figure 8. Boxplot showing the number of eggs laid by each female in the different treatments. The boxplot shows the 25- 75 % percentiles and the median which is marked with a black line within the box. Stars are showing outliers. Flies in the PDB control laid significantly fewer eggs compared to the treatments. We found no significant difference among the treatments. The flies in the H. uvarum treatment laid an average of 5,3 eggs, the flies in raspberry treatment 5,2 eggs, Raspberries plus H. uvarum 6,7 eggs and PDB 0,64 eggs. Means labelled with different letter are significantly different (p< 0.05; One-way ANOVA, Tukey's test; more statistical details are shown in Table 2).

A One- way ANOVA test followed by Tukey's simultaneous tests for differences of means was performed to see if there was a significant difference amongst the treatments and the control, results presented in Table 2. A 95% confidence level was used.

Table 2 Statistical analysis of eggs laid in the treatment. One-way ANOVA, Tukey's Simultaneous Tests for Differences of Means. The table shows the difference of means between the different treatments. SE of difference=The standard error of the difference between means. 95 % Cl stands for Confidence Interval for group means (95% CI). R= raspberry, Y= H.uvarum, R+Y= raspberries dipped in H. uvarum, PDB= Potato dextrose broth (control).

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
R+Y - R	1,43	1,27	(-1,88; 4,75)	1,13	0,671
PDB - R	-4,58	1,28	(-7,94; -1,23)	-3,57	0,003
Y - R	0,05	1,23	(-3,17; 3,27)	0,04	1,000
PDB - R+Y	-6,02	1,28	(-9,37; -2,66)	-4,69	0,000
Y - R+Y	-1,38	1,23	(-4,60; 1,84)	-1,12	0,676
Y - PDB	4,63	1,25	(1,37; 7,89)	3,72	0,002

Individual confidence level = 98,96%

When comparing the number of flies that laid one egg or more in the treatment compared to the ones not laying any eggs we found that the flies in the PDB control were less likely to oviposit compared to the three different treatments ($P = <0,05$, Fisher's exact test, correction according to Bonferroni). In the raspberry treatment 20 out of 23 (87 %) flies did oviposit, in raspberry plus *H. uvarum* treatment 19 of 23 flies oviposit (82,6 %) and in *H. uvarum* treatment 22 out of 26 flies oviposit (84,6 %) compared to the *PDB* control where only 3 of the 22 flies (13,6 %) did oviposit (see Figure 9. We found no significant difference between the number of flies that did oviposit between the different treatments ($P > 0,05$, Fisher's exact test, correction according to Bonferroni)
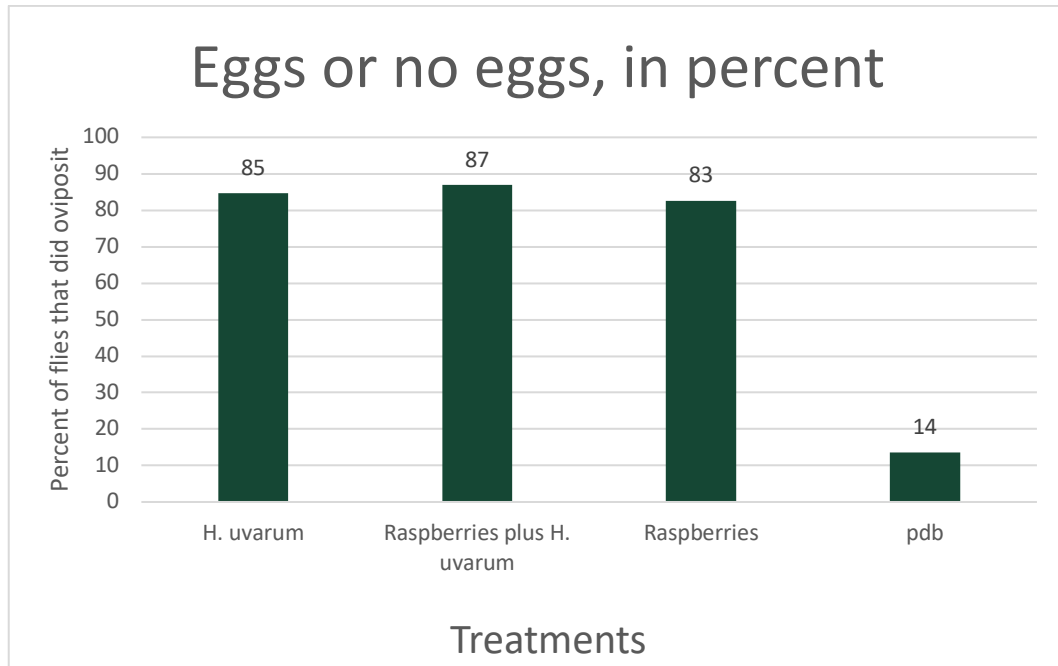
Figure 9. Percentage of the flies in each treatment that laid 1 or more eggs during the 24-hour experimental time (yeast n=26, raspberries n=23, raspberries plus H. uvarum n=23 and PDB n=22). The flies in the PDB control laid significantly fewer eggs compared to all the treatments. (P<0,05; Fisher's exact test, corrected with the Bonferroni method). We found no difference between the different treatments.

Fertility assessment

We found no difference in the number of eggs laid in the fertility assessment neither between the different treatment nor the control (Figure 10).

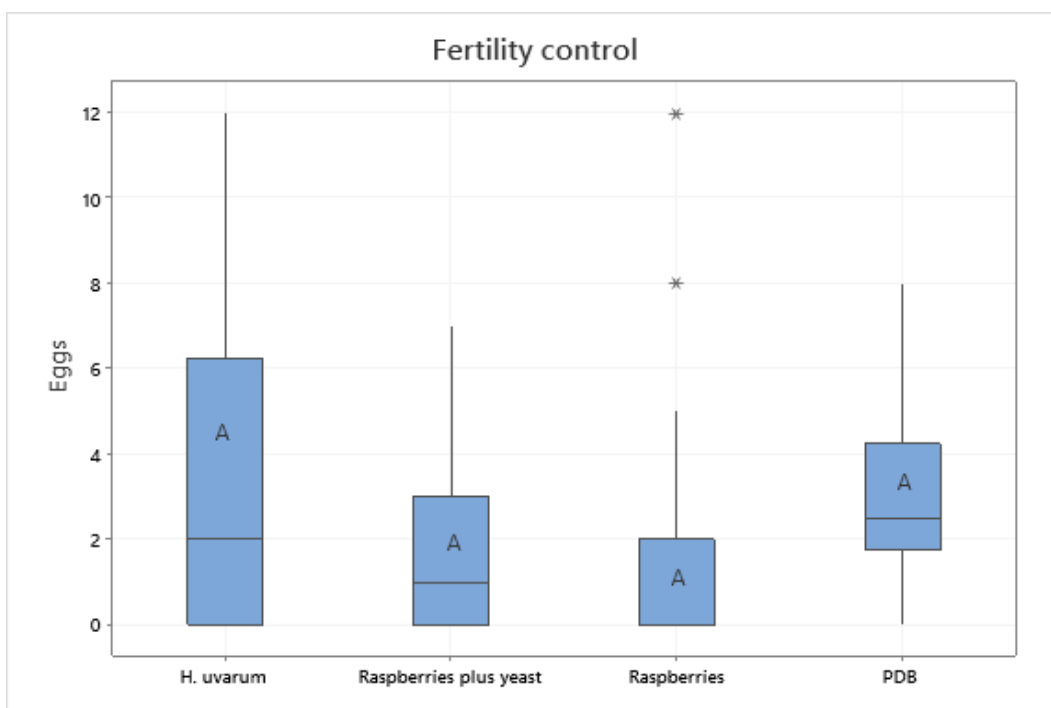*Figure 10. Boxplot showing the eggs laid by each female in the fertility assessment that we conducted immediately after the experiment. The boxplot shows the 25- 75 % percentiles and the median which is marked with a black line.Stars are showing outliers. In average the flies in the fertility control laid: H. uvarum 3,1 eggs/female (n=26), Raspberries 1,7 eggs/ female (n= 23), Raspberries plus 2 eggs/ female (n= 23) and PDB 3,1 eggs/ female (n = 22). We found no significant difference between neither the treatments nor the control, for statistical analysis see table 3 (One-way ANOVA, Tukey's test). Means that do not share a letter are significantly different.*

The statistical analysis of the eggs laid by each female in the fertility assessment is presented below. We found no significant difference between the different treatments or the PDB control for the eggs laid in the fertility assessment (P<0,05; One-way ANOVA, Tukey's test; Table 3)

Table 3. Statistical analysis of eggs laid in the fertility assessment analysed by One-way ANOVA and Tukey Simultaneous Tests for Differences of Means. The table shows the difference of means between the different treatments. SE of difference=The standard error of the difference between means. 95 % CI stands for Confidence Interval for group means (95% CI). R= raspberry, Y= H. uvarum, R+Y= raspberries dipped in H. uvarum, PDB= Potato dextrose broth (control)

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
R - Y	-1,463	0,811	(-3,584; 0,658)	-1,81	0,278
R+Y - Y	-1,115	0,811	(-3,236; 1,005)	-1,38	0,518
PDB - Y	-0,024	0,820	(-2,171; 2,122)	-0,03	1,000
R+Y - R	0,348	0,835	(-1,837; 2,533)	0,42	0,976

PDB - R	1,439	0,844	(-0,771; 3,648)	1,70	0,328
PDB – R+Y	1,091	0,844	(-1,118; 3,300)	1,29	0,571

Individual confidence level = 98,96%

Combination oviposition experiment and fertility assessment

When combining the data about eggs laid in the actual experiment and in the fertility assessment we found that the flies exposed to *PDB* in the experiment laid significantly less eggs in total (48 hrs) compared to the *H. uvarum* treatment and *H. uvarum* plus raspberry treatment, but not significantly less eggs compared to the raspberry treatment (Figure 11).
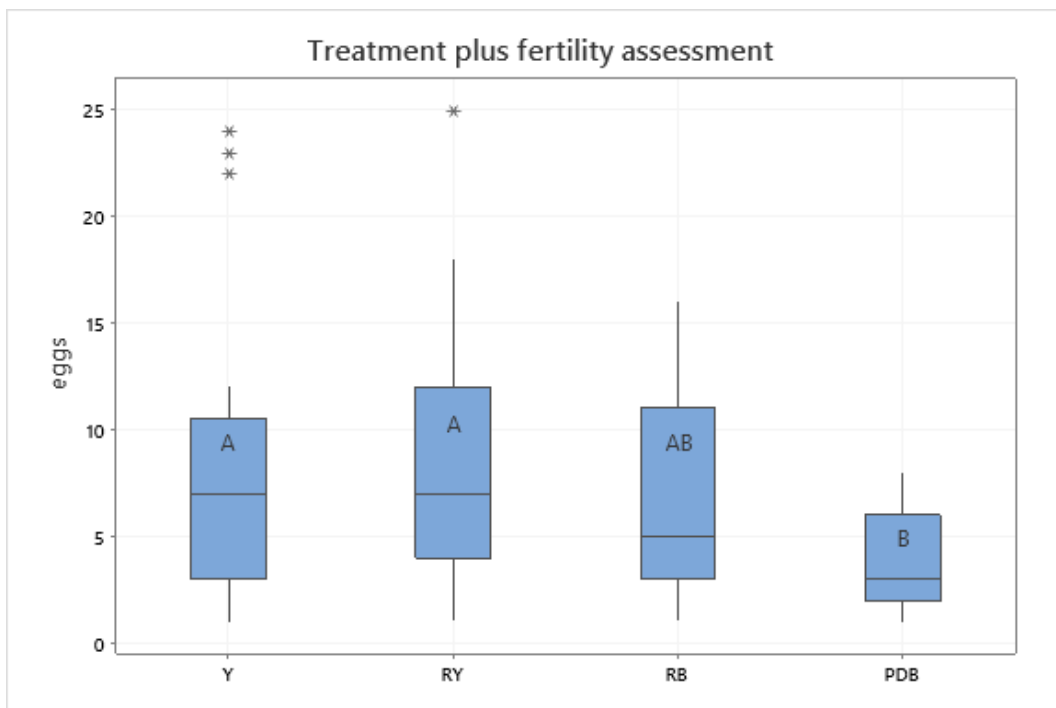

Figure 11. Boxplot showing the number of eggs laid over 48 hrs by the flies in the different treatments during the test and the subsequent fertility assessment. The boxplot shows the 25- 75 % percentiles and the median which is marked with a black line. Stars are showing outliers. The flies exposed to volatiles from H. uvarum and a combination of raspberries and H. uvarum laid significantly more eggs compared to the flies in the PDB control. These numbers were not significantly different to number of eggs laid by the flies exposed to raspberries (see Table 4). Means that do not share a letter are significantly different (One-way ANOVA, Tukey's test).

Statistical analysis for the eggs laid by each female when combining the experiment and the fertility assessment is presented in Table 4. One- way ANOVA followed with Tukey's pairwise comparisons was used.

Table 4 Statistical analysis of data on eggs laid in the treatment plus the fertility assessment analysed by One-way ANOVA and Tukey's Simultaneous Tests for Differences of Means. The table shows the difference of means between the different treatments. SE of difference=The standard error of the difference between means. 95 % Cl stands for Confidence Interval for group means (95% CI). R= raspberry, Y= H.uvarum, R+Y= raspberries dipped in H. uvarum, PDB= Potato dextrose broth (control).

Difference of Levels	Difference of Means	SE of Difference	95% CI	T-Value	Adjusted P-Value
RY - Y	0,27	1,44	(-3,51; 4,04)	0,19	0,998
RB - Y	-1,52	1,44	(-5,29; 2,26)	-1,05	0,720
PDB - Y	-4,66	1,46	(-8,48; -0,84)	-3,19	0,010
RB - RY	-1,78	1,49	(-5,67; 2,11)	-1,20	0,629
PDB - RY	-4,92	1,50	(-8,86; -0,99)	-3,28	0,008
PDB - RB	-3,14	1,50	(-7,07; 0,79)	-2,09	0,164

Individual confidence level = 98,96%

4. Discussion

The association between *D. suzukii* and *H. uvarum* is well known but the ecological significance of this interaction is not fully understood. In this thesis, we have been looking at the importance of volatiles emitted by *H. uvarum* when finding a place to mate and oviposit for *D. suzukii.* Reproduction has a fitness cost and might for example cause shorter life length or wounds in female *drosophilids* (Smith 1958; Chapman et al 1995; Kamimura 2007). Since females fed with yeast lay more eggs and yeast is crucial for larval development (Bellutti et al. 2018) feeding and mating at a place where yeast is available might have positive effects on offspring survival.

4.1. Mating assay

In the well-studied model organism, and close *D. suzukii* relative, *D. melanogaster*, volatiles from food had been found to induce mating in both males and females through ionotropic receptors which are part of the olfactory system (Grosjean 2011; Gorter et al, 2016). Gorter et al (2016) found that *D. melanogaster* mated more times during a 24 hrs period when yeast was present compared to the ones not exposed to yeast which was dependent on the female ionotropic receptor Ir75a sensing acetic acid, a common compound in yeast. Our result indicates that for *D. suzukii,* similar to *D. melanogaster* food odour might induce mating. In our experiment, virgin couples of *D. suzukii* exposed to volatiles from *H. uvarum* were more likely to mate during the 30 minutes experiment and started to mate during the whole experimental time compared to the flies in the blank control that mostly mated at the beginning of the experiment. Gorter et al (2016) did not get any significant increase in mating with only volatiles, in their experiment the yeast needed to be present to the flies to induce mating. They suggest that it is the

combination of the hedonic value from yeast volatiles and the nutritional value of the yeast that increases the mating. In our experiment volatiles from *H. uvarum* seem to elicit mating in virgin flies, which is in favour of the hedonic value from the food odours for *D. suzukii*. However, in our experiment the flies were picked directly from vials with yeast containing food, if starved they might have responded differently.

In subsequent control experiments, exposing flies to water or PDB, we did not see any difference in mating compared to flies exposed to *H. uvarum*. This inconsistency may be due to a limited number of replicates, in this experiment we only tested about 20 couples in each treatment (water n= 20, PDB n= 20 and *H. uvarum* n= 22), compared to 48 (yeast) and 40 (blank) in the previous assay. Importantly, the overall lower mating rate in the control experiments suggest that the test did not perform optimally. A possible explanation for this might be that this assay was conducted at another season- summer instead of early spring, which might have an impact even though the experiment was conducted in a controlled environment with artificial daylight. However, these results indicate that humidity might need to be considered when further investigating this issue. Higher relative humidity has been shown to increase the reproductive potential in *D. suzukii* (Tochen et al. 2016b). For future experiments, it would be interesting to include volatiles from host fruits, which in previous studies has been shown to induce mating in *D. melanogaster* males (Groesjean et al. 2011) to further understand which volatiles affecting mating behaviour in *D. suzukii*.

4.2. Oviposition assay

D. suzukii uses visual and tactile cues to select a place to oviposit (Kirkpatrick et al, 2015; Rice et al. 2016; Little et al. 2018; Entling et al. 2018). Our experiment, which was conducted in darkness, shows that even when removing visual cues, volatile cues are enough to induce oviposition. Volatiles from ripe raspberries evokes

oviposition in *D. suzukii*, which is supported by previous research from Karageorgi et al. 2017. We found that the flies exposed to volatiles from *H. uvarum* did oviposit to an equal extent as the ones exposed to fruit odour. This can be compared to *D. melanogaster*, where yeast *Saccharomyces cerevisiae* has been shown to provoke oviposition (Becher et al. 2012*)* and volatiles emitted by fresh fruit seem to be less stimulating (Karageorgi et al. 2017). This difference between the species is in agreement with their niches – *D. suzukii* oviposits both on ripe and decaying fruit compared to *D. melanogaster* which oviposits on decaying fruit (Iglesias et al. 2014; Hamby & Becher 2016).

Volatile cues from fruit or yeast seem to be important when deciding to lay eggs or not, as very few of the flies in the PDB control did oviposit and laid significantly fewer eggs compared to all treatments. The flies in the PDB control laid a similar amount of eggs in the fertility assessment compared to the flies from the other treatments. This shows that the flies in the PDB control consequently retained their eggs until they were offered a more preferred substrate in the fertility assessment. When combining the eggs laid in the experiment and the fertility assessment (48 hrs) the flies in the PDB treatment laid significantly fewer eggs in total compared to the flies exposed to *H. uvarum* in the experiment, a difference not significant to the flies only exposed to raspberries. Accordingly, flies in the PDB control, that laid very few eggs during the 24 hrs in the experiment, were not able to fully compensate by increased egg-laying in the subsequent 24 hrs when exposed to a more suitable oviposition substrate.

In a pilot study (data not shown) where we tested the same setup, using fresh blueberries as a volatile cue instead of raspberries, the flies did not oviposit in this treatment but in the *H. uvarum* treatment. When we used blueberries with cuttings, left to decay overnight, the flies started to oviposit. This could be due to the difference in volatile emissions from undamaged blueberries compared to raspberries. However, there might be a chance natural occurrence of microbes

colonizing the raspberries contribute to the attraction even in the fresh berries. For further studies, one could look at what chemical compounds in yeast and fruit volatiles that is connected to oviposition.

4.3. Microbe association and invasion biology

Karageorgi et al. (2017) studied three *Drosophila* species; *D. melanogaster, D. biarmipes,* and *D. suzukii,* and found that *D. suzukii* preferred ripening fruit, *D. melanogaster* preferred decaying fruit, and *D. biarmipes* was found to be in between. This could be explained by an evolutionary path where attraction to ripening fruit becomes stronger with the use of maturing fruit as a substrate for oviposition and larval development. *D. suzukii* and *D. biarmipes* also seem to have a stronger attraction to volatiles from green leaves compared to *D. melanogaster* (Keesey et al. 2015), which can be one explanation they get attracted to the cultivation before fruit ripens. The success of *D. suzukii* as a pest of ripening fruit might be a combination of an ancestor linage that prefers ripening fruits combined with the development of the serrated ovipositor (Karageorgi et al. 2017; Atallah et al. 2014). Female *D. suzukii* tolerates stiffer oviposition substrates compared to *D. biarmipes* and *D. melanogaster* (Karageorgi et al. 2017). The niche of ripening fruit could be used to decrease competition with other drosophilids. *Drosophila* preference to feed on different yeast sources is important for species differentiation and a way for different drosophilids to share a common resource (Starmer & Phaff 1983; Becher et al. 2012). Even if *D. suzukii* developed a niche to oviposit in ripening fruit, yeasts are still an important part of their lifecycle and they are highly attracted to yeast (Iglesias et al. 2014; Scheidler et al. 2015). *H. uvarum* is the yeast generally available in the early stages of fermentation and therefore might be the only or one of few yeasts available for *D. suzukii* larvae (Schütz 1993; Ciani et al. 2006; Hong & Park 2013). Microbes are known to facilitate the invasion biology of invasive species (Lu et al. 2016). The

association with the early stage fermenting yeast *H. uvarum* is one among other factors, that might make *D. suzukii* such a successful pest.

4.4. Microbial volatiles in plant protection

Like other drosophilids *D. suzukii* has an association with a specific yeast flora where *H. uvarum* is the predominant one (Hamby et al. 2012). *D. suzukii* shows species-specific attraction for *H. uvarum* compared to other yeasts (Scheidler et al. 2015). This suggests that *H. uvarum* could be used in integrated pest management. A species-specific trap baited with *H. uvarum* can be helpful for the farmers not having to distinguish different *Drosophila* species from each other, which can be challenging and complicate monitoring (Burrack et al. 2015). When placing *H. uvarum* baited traps the possibility to attract flies from the surrounding area should be considered. In a laboratory study, *H. uvarum* applied on grape leaves was similarly attractive to mated *D. suzukii* as fresh grapes (Rehermann et al. in prep). This indicates that *H. uvarum* could be used, for trapping or mixed with an insecticide to target *D. suzukii* even in cultivation with ripe fruit present. The figure below shows how *D. suzukii* interaction with microbes might look like in cultivation and how microbes, in a trap or mixed with an insecticide might be used for pest management.
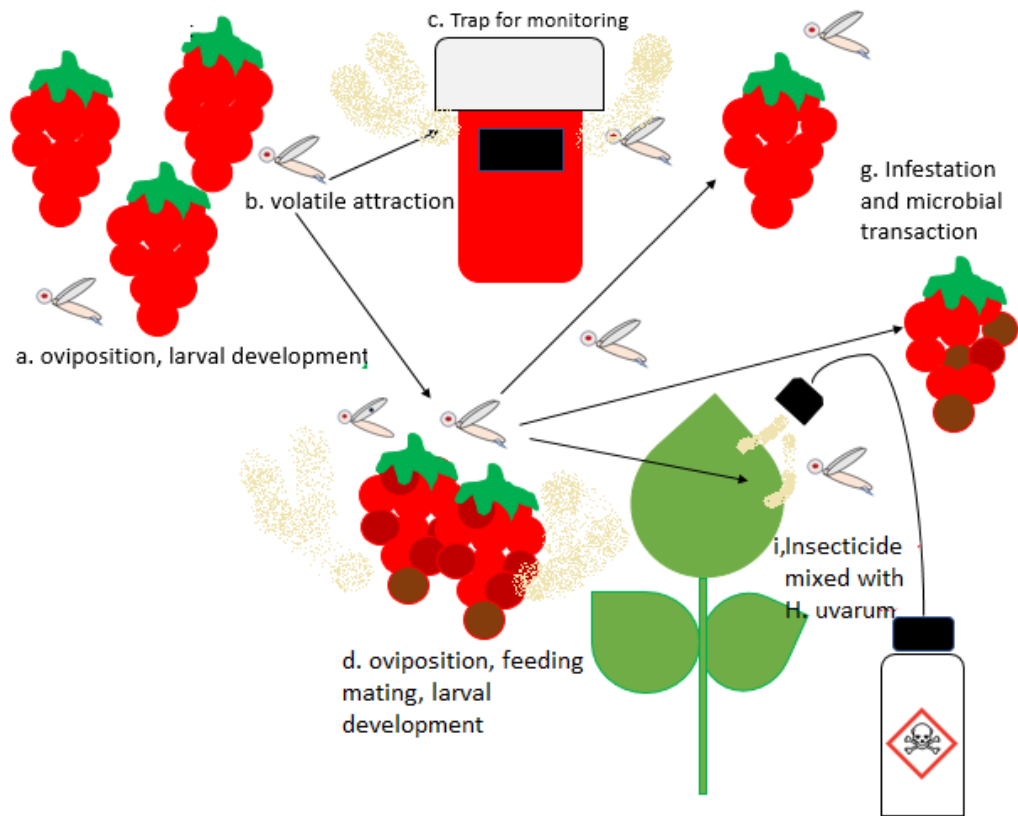
Figure 14. Illustration of Drosophila suzukii and microbe interactions and possible application of microbes in pest control management a, Drosphila suzukii oviposit and larvae develop in ripe fruit, b, volatile attraction to food volatiles, like H. uvarum lead D. suzukii to either c; trap for monitoring or d; overripe fruit where D. suzukii fed, lay eggs and mate on the surface of the overripe fruit. Mated D. suzukii moves to new ripe or overripe fruit (g) for oviposition and at the same time vectors microbes or to (i) a pesticide mixed with H. uvarum applied on the leaf surface. Repainted with slight modifications from Hamby and Becher (2016).

An uncontrolled infestation of *D. suzukii* may cause major economic loss in the cultivation of soft skin fruit and berries (Walsh et al. 2011; Bolda et al. 2010; Illoratti 2011). Finding effective and specific ways to target *D. suzukii* is important.

4.5. Conclusions

This study suggests that volatiles from *H. uvarum* stimulate mating in virgin *D. suzukii* couples compared to a blank control. Relative humidity should be considered if replicating this experiment.

In our experiment volatiles from the yeast *H. uvarum*, fresh raspberries, or a combination of these, stimulated oviposition in *D. suzukii* to an equal and significantly higher extent compared to the control.

Gravid flies in the control treatment in the oviposition experiment, who did not sense either fruit or yeast volatiles for the first 24 hrs after mating, seemed to be able to, and to a high extent choose to contain their eggs for at least 24 hrs, until exposed to fruit were they did oviposit.

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