Project title:	**The use of highly attractive yeast strains for controlling *Drosophila suzukii* (spotted wing drosophila).**
Project number:	CP 171
Project leaders:	Rory Jones (author) Michelle Fountain (NIAB EMR) Matthew Goddard (University of Lincoln)
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Location of project:	NIAB EMR and University of Lincoln
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AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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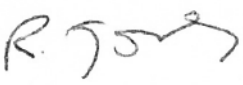
Signature  Date 25 September 2019

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GROWER SUMMARY

Headline

Four combinations of yeast have been found as attractive to *D. suzukii* in laboratory choice tests, three of which are also attractive in the field. Additionally, three single yeasts previously shown to be attractive in the laboratory in the previous year of this project also proved to be attractive in the field. Work has begun characterising the microbial communities on fruit as they ripen.

Background

Since being identified in the UK, in 2012, *Drosophila suzukii* has caused considerable damage to commercial fruit resulting in yield losses and increasing expenditure on control methods. Currently *D. suzukii* is controlled through the use of plant protection products, crop hygiene measures and insect exclusion mesh. With more stringent measures being increasingly implemented on the use of plant protection products, often resulting in the withdrawal of particular products combined with the threat of insecticide resistance from a limited number of active ingredients, new control methods need to be developed and optimised.

There are complex interactions between fruit, microbes and *Drosophila* species, and understanding these is important for the control of *D. suzukii*. Yeasts are an essential source of nutrients for *Drosophila*; they are important for oviposition and larval development. Some yeast species, most notably *Hanseniaspora uvarum,* are attractive to *D. suzukii* and have the potential to produce highly attractive and selective baits. There are two potential approaches for yeast in *D. suzukii* control.

Firstly, precision monitoring, where numerous traps capture adult *D. suzukii.* This is widely available and easy to implement although it is labour-intensive. To date, this method has not been demonstrated to reduce crop damage. Trapping is recommended for the monitoring and detection of *D. suzukii* and lure-and-kill strategies could be used in integrated pest management of *D. suzukii*. However, more attractive and selective baits are needed to reduce the capture rates of non-target species. This would also make detecting *D. suzukii* females easier as they can be mistakenly identified for other *Drosophila* species without the aid of a microscope.

Secondly, attract-and-kill strategies, which combine yeast with plant protection products thus attracting flies to kill them, could be part of an IPM programme. This may enable a reduction in the amount of synthetic plant protection products applied whilst simultaneously increasing the targeted exposure of *D. suzukii.* This could reduce exposure of non-target species to plant protection products and reduce residues on fruit. A study of the literature and AHDB project SF 145 have both shown that combining plant protection products with the yeast species *H. uvarum* increases mortality and reduces egg laying compared to plant protection products alone.

Yeast has been used to trap *Drosophila* for many years; typically, dried baker's yeast has been used in fermentation-based baits. Recently, since 2012, there has been a focus on the potential use of *H. uvarum*, which is associated with *D. suzukii*, for controlling this pest. Although, *H. uvarum* is known to be attractive to *D. suzukii* not many other yeast species have been tested for attractiveness. This project will not only test the attractiveness of yeasts from an existing culture collection but also yeasts that will be isolated from ripening fruit (strawberries, raspberries, blueberries and cherries). Unlike the majority of *Drosophila* species, *D. suzukii* oviposit in ripening fruit, therefore yeast from ripening fruit may not only be attractive but selectively attractive to *D. suzukii.* In nature, microbes on the surface of fruit are complex and, currently, only single yeasts have been tested for attractiveness. This project will also test the attractiveness of combinations of yeasts.

The main aim of this project is to identify highly attractive yeast species alone and in combination, and then utilise these in the control of *D. suzukii.* Additionally, this project will aim to characterise microbial communities on ripening fruit and investigate identified yeast for attraction to *D. suzukii* as well as ability for use in control strategies.

Summary

Yeasts vary in their attraction to *Drosophila* species and previous work in 2017/18 identified four candidate yeast species attractive to *D. suzukii*: *H. uvarum*, yeast coded 218, 164, and 190*. D. simulans* was indifferent to all four but *D. melanogaster* was attracted to all four. Both are common non-target species often captured in *D. suzukii* monitoring traps; highlighting the potential for yeast to produce attractive and selective baits for *D. suzukii.* Additionally, multiple strains of *H. uvarum* were attractive to *D. suzukii*. This is a yeast species that, in the context of *D. suzukii,* has received a lot of attention in the literature and is known to be attractive to *D. suzukii*, both in the literature and project SF 145. Building on this work, we tested the attractiveness of ferments of these yeasts in the field using standard commercially available traps. Three yeasts were attractive in the field, yeast coded 190, and two strains of *H. uvarum*.

In addition, combinations of yeasts were screened, both in the laboratory and the field. Of the combinations tested, four proved to be significantly attractive in the laboratory; 201+164, 190+201, 190+218+201, and 190+218, but in the field only the latter three combinations attracted *D. suzukii*. Currently, none of the yeast-based attractants tested in the field proved significantly more attractive than commercial Gasser lure. It is worth noting that these field-based trials were conducted between late October and early December.

Microbial communities on ripening fruit are also currently being investigated. Fruit samples were collected in 2018 from four ripening stages of blueberries, cherries, strawberries and raspberries. Fruits were surface washed to collect microbes; DNA was extracted and ITS regions (conserved across fungal species) were amplified and sequenced. Cherry and blueberry samples have been sequenced, and analyses begun. However, the strawberry and raspberry samples have only recently been sent for sequencing. Once all data is collated this will be analysed using 2-way Permanova for both presence/absence as well as abundance, indicator species and UniFrac analysis. Preliminary analyses have revealed that for cherries there was a significant effect of ripening stage on fungal species (OTUs) present, but for blueberries this was not the case.

Financial Benefits

D. suzukii is an economically damaging pest that causes loss to soft and stone fruit yields. This project has the potential to improve *D. suzukii* control and reduce residues in fruits. The attractive yeast species and strains identified by this project could potentially be exploited in monitoring and control of *D. suzukii* in IPM strategies to more effectively combat this pest.

Action Points

There are currently no action points that growers need to implement at this stage.

SCIENCE SECTION

Introduction

Drosophila suzukii (Matsumra) is a highly polyphagous pest that is endemic to Southeast Asia and has recently spread around the globe. In 2008 *D. suzukii* was first detected on the American mainland in California (Bolda *et al.,* 2010). *Drosophila suzukii* then spread quickly to many other countries and is now also present in most northern temperate regions (Rota-Stabelli *et al.,* 2013; Asplen *et al.,* 2015). It was first detected in the UK in 2012 (Harris and Shaw, 2014). It is likely that *D. suzukii's* global spread is due to human-aided transportation (Hauser, 2011; Calabria *et al.,* 2012). Since its recent spread around the globe, *D. suzukii* has caused massive economic losses. Total losses attributed to *D. suzukii* in 2008 in three states in the USA in strawberries, blueberries, raspberries, blackberries and cherries totalled $511.3 million (Bolda *et al.,* 2010). In Europe, *D. suzukii* causes substantial losses, with 80% losses to strawberry crops in a region in the south of France and between 60-80% losses of strawberry crops, 90% losses of late-harvested cherries and 90-100% of blueberry crops being affected in some regions in Italy in 2010 (Grassi *et al.,* 2011). Unlike most *Drosophila* species, *D. suzukii* oviposits in ripening fruit; this ability derives from female *D. suzukii* having a morphologically modified ovipositor (Atallah *et al.,* 2014). Oviposition can lead to mechanical damage in the form of puncture wounds in the surface of fruit. Once the larvae hatch they cause additional mechanical damage by feeding, resulting in unmarketable fruit (Goodhue *et al.,* 2011). The open wounds inflicted on the fruit allow secondary infection by bacteria and yeast species (Loriatti *et al.,* 2017). In addition to this, the damage inflicted on fruit by *D. suzukii* allows oviposition entry by other insects that lack the ability to oviposit in undamaged ripe fruit (Walsh *et al.,* 2011).

Microbes associated with fruit

Microbes are essential components of agricultural and natural ecosystems. Fruits provide conditions that support microbial growth with good water availability and access to sugars and other nutrients (Berg *et al.,* 2016). Microbes inhabiting the surface of plants form complex communities whilst fulfilling a variety of roles, some beneficial, some neutral and some pathogenic to plants. Several factors have an effect on fruit microbial communities; geographic location (Gayevskiy and Goddard, 2012; Taylor *et al.,* 2014; Bokulich *et al.,* 2014), plant organ, fruit ripening stage (Barata *et al.,* 2012), farming practices (Martins *et al.,* 2014), fruit species (Leff and Fierer, 2013) and even fruit variety (Cordero-Bueso *et al.,* 2011;

Gayevskiy and Goddard 2012). Some studies reported little variation between fungal communities and geographic region in some fruit species but variation in others (Vepštaitė-Monstavičė *et al.,* 2018). Niche generally explains more variance in fungal communities than geographic location (Morrison-Whittle and Goddard, 2015).

Yeasts, which are single-celled fungi, colonise various surfaces of plants and are more abundant and have greater community diversity on fruits compared to blossoms (Vadkertiová *et al.,* 2012). A small number of abundant taxa dominate yeast communities on fruit (Hamby *et al.,* 2012). *Hanseniaspora uvarum*, *Hanseniaspora guilliermondii*, *Metschnikowia pulcherrima* and *Pichia kluyveri* were regularly isolated from various fruit surfaces; including apples, plums and pears using culture-based methods (Vadkertiová *et al.,* 2012). Apples are dominated by the fungal phylum Ascomycota and Basidiomycota (Vepštaitė-Monstavičė *et al.,* 2018). Analogous to bacterial populations on apples, little difference was detected between geographic location and fungal population on apples (Vepštaitė-Monstavičė *et al.,* 2018). Yeast communities change over time on nectarines (Janisiewicz *et al.,* 2010), plums (Janisiewicz *et al.,* 2014) and grapes (Martins *et al.,* 2014). Strawberries harbour a wide variety of fungal taxa (Abdelfattah *et al.,* 2016). Similar to apples, the diversity of fungal communities on strawberries differs significantly with fruit maturity and between different parts of the plant including, flowers and leaves (Abdelfattah *et al.,* 2016).

Yeasts associated with Drosophila.

Drosophila are saprotrophic and thus dependent on microbes for nutrition. Microbes are required for the development of both *D. suzukii* and *D. melanogaster* (Meigen) (Bing *et al.,* 2018). *Drosophila* are attracted to yeasts (Dobzhansky *et al.,* 1956) and yeast is used in fermentation baits to catch *Drosophila*. Female flies prefer to oviposit on yeast-colonised substrates (Oakeshott *et al.,* 1989), resulting in an increased development rate of larvae (Meshrif *et al.,* 2016; Bellutti *et al.,* 2018). The same yeast lineages are often present in *Drosophila* irrespective of species, geographical location or diet (Chandler *et al.,* 2012). In *D. melanogaster* survival rate and development time vary with yeast species availability (Meshrif *et al.,* 2016). Giving *D. melanogaster* larvae access to either *Pichia toletana* or *Metschnikowia pulcherrima* decreased development time whilst simultaneously increasing survival rates. *Pichia toletana* was more beneficial than *M. pulcherrima*; in addition, choice tests revealed that *P. toletana* was more attractive to *D. melanogaster* larvae (Meshrif *et al.,* 2016). Both larvae and adult *D. melanogaster* engage in niche construction, manipulating yeast communities to differing degrees (Stamps *et al.,* 2012). Yeasts are immobile and therefore

rely, in part, on insects to transport them to new habitats. *Drosophila* adults may vector yeast, potentially inoculating yeast onto new fruit surfaces (Gilbert, 1980; Starmer *et al.,* 1987; Stamps *et al.,* 2012; Buser *et al.,* 2014) with a significant effect on communities (Stamps *et al.,* 2012) and *Drosophila* have been shown to vector yeast in the laboratory (Christiaens *et al.,* 2014; Buser *et al.,* 2014). There are however limited studies demonstrating this in nature, but a strain of *Saccharomyces cerevisiae* that is attractive to *D. simulans* has been shown to be dispersed more frequently than an unattractive strain of *S. cerevisiae* in a vineyard setting (Buser *et al.,* 2014). Additionally, flies had higher fecundity when associated with more attractive yeasts (Buser *et al.,* 2014) and thus these studies provide some evidence to support the existence of a mutualism between yeast and *Drosophila* as both gain fitness advantages by interacting (Christiaens *et al.,* 2014; Buser *et al.,* 2014), but there is not enough evidence to assume that this mutualism extends to hold generally between yeast and *Drosophila* (Günther and Goddard, 2018). In addition, larvae have considerable effects on yeast species composition, reducing yeast diversity on fruits (Stamps *et al.,* 2012). Larvae and adults from four different *Drosophila* species had a preference for certain yeast species when given the choice; this preference differed between larvae and adults of the same species (Cooper, 1960). Stamps *et al.* (2012) also demonstrated that *D. melanogaster* larvae consistently encouraged the development of yeast communities comprised of three yeast species; *Candia cailfornica*, *Candidia zemplina* and *P. kluyveri* (Stamps *et al.,* 2012).

Yeast species vary in attractiveness to *D. melanogaster* with *Saccharomyces* species generally being more attractive than non-*Saccharomyces* species (Palanca *et al.,* 2013). Additionally, yeasts isolated from fruit were more attractive than non-fruit isolated yeast (Palanca *et al.,* 2013). *Saccharomyces cerevisiae* strains also vary in attractiveness to both *D. melanogaster* (Palanca *et al.,* 2013; Schiabor *et al.,* 2014) and *D. simulans* (Sturtevant) (Buser *et al.,* 2014). Only single yeasts have been tested for attractiveness to *Drosophila* species (e.g. Palanca *et al.,* 2013; Buser *et al.,* 2014; Günther *et al.,* 2015; *Scheidler et al.,* 2015). It is not known whether there is a synergistic or additive effect of combinations of different yeasts, as occurs in nature, on attractiveness. In reality, microbial communities on fruit are much more complex (e.g. Taylor *et al.,* 2014). Yeast combinations warrant further investigation and may have the potential to produce both attractive and selective baits.

Drosophila suzukii and yeast.

There are complex interactions between fruit, microbes and *Drosophila* species and understanding these interactions is important in the control of *D. suzukii* (Hamby and Becher, 2016). Yeast have been utilised for trapping *Drosophila* for a long time (e.g. Da Cunha *et al.,*

1951). Yeast can be exploited in baits to attract *D. suzukii,* with baits incorporating yeast capturing more *D. suzukii* than baits like apple cider vinegar (Iglesias *et al.,* 2014). *D. suzukii* is associated with the yeast species *H. uvarum*; one study found, from the total 28 yeast species identified, *H. uvarum* was the dominant yeast species isolated from both *D. suzukii* larvae and adults, followed by *Issatchenkia terricola* and *P. kluyveri* (Hamby *et al.,* 2012). Non-Saccharomyces yeasts from the genera *Hanseniaspora*, *Pichia* and *Candida* have also been identified from the guts of adult winter morph *D. suzukii* (Fountain *et al.,* 2018). Hamby et al. (2012) affirmed that as the abundance of *H. uvarum* was higher in *D. suzukii* compared to other *Drosophila* species (Heed *et al.,* 1976) this could mean that there was a specific association between *D. suzukii* and *H. uvarum* making it an ideal candidate for a yeast-based bait (Hamby *et al.,* 2012). If this is the case, it could be fundamentally important in the creation of selective and attractive bait for *D. suzukii.* In addition, *H. uvarum* was attractive to *D. suzukii* when tested against multiple yeast species (Scheidler *et al.,* 2015). However, *H. uvarum* is common in the environment and on fruits. *Hanseniaspora uvarum* has been isolated from cherries, raspberries (Hamby *et al.,* 2012), apples, plum, pears (Vadkertiová *et al.,* 2012) and grapes (Barata *et al.,* 2012; Gayevisky and Goddard, 2012). *H. uvarum* is also attractive to other *Drosophila* species, including *D. melanogaster* (Scheidler *et al.,* 2015; Palanca *et al.,* 2013; Hoang *et al.,* 2015), making it unlikely to be a species-specific relationship. However, as *H. uvarum* is attractive to *D. suzukii* (Scheidler *et al.,* 2015; Noble *et al.,* 2019) it is still an important candidate yeast which warrants future investigation into its attractiveness to *D. suzukii*.

Drosophila suzukii control utilising yeast

Currently *D. suzukii* is controlled through the use of plant protection products, crop hygiene measures and insect exclusion mesh. With more stringent measures being increasingly implemented on the use of plant protection products, often resulting in the withdrawal of particular products combined with the threat of insecticide resistance from a limited number of active ingredients; new control methods need to be developed and improved.

There are two potential approaches for yeast in *D. suzukii* control. Firstly, mass trapping is a relatively cheap method of control which is widely available and easily implementable, but this method can be labour-intensive (Lee *et al.,* 2011). Trapping is essential for the monitoring and detection of *D. suzukii*, and greater incidence of *D. suzukii* has been recorded in spring when fruit infestation levels were low and vice versa in early July. This suggests lures may be less attractive than ripening fruit (Beers *et al.,* 2011) highlighting the need to develop more attractive and selective baits. Lure-and-kill strategies using traps are very important

candidates in the IPM management of *D. suzukii*. However, for mass trapping more attractive and selective baits are needed to reduce the capture rates of non-target species; this would also make detecting *D. suzukii* females easier as they can be mistakenly identified for other *Drosophila* species without the aid of a microscope. Therefore, reducing the numbers of other *Drosophila* species in traps with a bait that is selective to *D. suzukii* would make it easier to process trap captures (Hamby *et al.,* 2012). Secondly, one promising avenue of research is attract-and-kill strategies which combine yeast with pesticides thus attracting flies to kill them (Hamby and Becher, 2016; Mori *et al.,* 2016; Noble *et al.,* 2019; SF145). This may enable a reduction of the amount of synthetic pesticide input generally whilst simultaneously increasing the targeted exposure of *D. suzukii.* Importantly, this means it may reduce the exposure to non-target species to pesticides and reduce residues on fruit. *Drosophila suzukii* females lay fewer eggs and mortality increases when they are exposed to *H. uvarum* combined with the Spinosad pesticide (Mori *et al.,* 2017) demonstrating the potential for utilising yeast combined with pesticides for control.

Aims

In this project the following null hypotheses will be tested:

H1-There is no significant difference between the attractiveness of different yeast species to different *Drosophila* species *(D. melanogaster*, *D. simulans* and *D. suzukii)*.

H2-There is no significant difference between the attractiveness of different *H. uvarum* strains to *D. suzukii*.

H3-The effect of combining yeast on the attraction of *D. suzukii* will follow an additive (linear) model i.e. there will be no interaction effect or synergistic effect of combining yeast on attraction.

H4-There are no significant differences in the yeast communities on ripening fruit, regardless of fruit type or stage of ripening.

H5-There is no significant difference between highly attractive yeasts or yeast combinations to *D. suzukii* in comparison to currently available commercial baits.

The aims addressed by this report were;

1. Test attractiveness and repulsiveness of individual yeast species to *D. suzukii* in the field and combinations of yeast both in the laboratory and field.
2. Characterise yeast communities on ripening fruit, strawberries, raspberries, blueberries and cherries.

Materials and methods

Drosophila maintenance

D. suzukii cultures were derived from an Italian strain, which was established at NIAB EMR in 2013. Summer morph *D. suzukii* populations were housed in BugDorm cages (32.5 x 32.5 x 32.5 cm) (MegaView Science Co., Ltd.) situated in a temperature-controlled room, which provided a constant temperature of 25**°**C and a 16:8 h light: dark photoperiod (Shaw *et al.,* 2018). Damp blue absorbent paper was placed on the base and roof of the cages to provide humidity (96%) inside the cages. *D. suzukii* were fed on Drosophila Quick Mix Medium blue (Blades Biological Ltd) sprinkled with dried baker's yeast and were also cultured on media comprising of 100% distilled H_20, 1% agar, 9% sugar, 9% pre-cooked ground maize, 2% baker's yeast, 5% malt 1% soy flour, 0.3% propionic acid and 0.3% Methylparaben (methyl 4-hydroxybenzoate) pre-dissolved in 70% ethanol (as per Shaw *et al.,* 2018). Additionally, *D. suzukii* were also regularly provided with frozen raspberries (Shaw *et al.,* 2018).

Yeast cultures

Yeast species were cultured from the Goddard culture collection, University of Lincoln, stored on glycerol at -80°C, originating from different locations including New Zealand, UK and USA (Table 1).

Table1: Origin, source and strain of yeast species used in choice tests.

Yeast Strain	Origin	Source	Reference
EC-1118	France	Commercial wine yeast	Lallemand Inc.
162	New Zealand	Chardonnay juice	Anfang *et al.,* 2009.
218	New Zealand	Pinot noir ferment	Goddard culture collection
150	New Zealand	Beehive	Anfang *et al.,* 2009.
164	New Zealand	Chardonnay ferment	
212	New Zealand	Syrah fruit	Gayevskiy *et al.,* 2012.
198	New Zealand	Sauvignon Blanc ferment	Goddard culture collection
190	New Zealand	Sauvignon Blanc ferment	Goddard culture collection
166	New Zealand	Sauvignon Blanc ferment	Goddard culture collection
98-3	United Kingdom	*D. subobscura*	Goddard culture collection
44-1	United Kingdom	*D. subobscura*	
201	New Zealand	Chardonnay fruit	Gayevskiy *et al.,* 2012.
206	New Zealand	Chardonnay fruit	Goddard culture collection
209	New Zealand	Chardonnay fruit	
11-382	United States	*D. suzukii*	Phaff Yeast culture collection, UC-Davis
44-9	United Kingdom	*D. subobscura*	Goddard culture collection
28-1	United Kingdom	*Drosophila* sp.	
28-5	United Kingdom	*Drosophila* sp.	
28-9	United Kingdom	*Drosophila* sp.	

Ferment Preparation

A preculture was prepared using YPD (yeast extract 1%, peptone 2%, dextrose 2%) media. Individual yeast species were transferred into 15 ml falcon tube containing 3 ml of YPD media. This was incubated at 30°C and 180rpm for 24 hours; the optical density was ascertained after inoculation and at 24 hours using a spectrophotometer (Jenway 6705). The ferments were then created by inoculating sterile strawberry juice with approximately 10^5 per ml yeast cells (Buser *et al.,* 2014, Günther *et al.,* 2015). Strawberry juice was cold-sterilised by filtration, where juice was passaged through a 0.2 µL filter system (Corning 1L Filter system 0.2 µL) laboratory assays or sterilised using up to a maximum of 1000 µl dimethyl dicarbonate (DMDC) dissolved in ethanol at a ratio of 1:2 DMDC to ethanol per litre of fruit juice for field

assays. Prior to being subjected to filtration, strawberry juice was pre-filtered through two layers of muslin to help reduce clogging of the filter which could potentially reduce efficiency of the filtration process. The brix and optical density (OD) of the yeast ferments were determined at 48 hours. The brix was ascertained using a refractometer (HI 96801, Hanna instruments) and the OD was again measured using a spectrophotometer. One ferment was used per yeast, so as to reduce variation.

Choice tests

To ascertain the attractiveness of yeast species, two-way choice test experiments were carried out. Choice tests using T-maze, or similar variants, are often utilised apparatus to determine preference to various olfactory stimuli in *Drosophila* (e.g. Begg and Hogben 1946; Palanca *et al.,* 2013; Buser *et al.,* 2014; Günther *et al.,* 2015).

Two-way choice tests using multiple T-maze apparatus were employed to ascertain the attractiveness of yeasts (Palanca *et al.,* 2013; Buser *et al.,* 2014; Günther *et al.,* 2019). Situated at each side of the T-Maze apparatus was 10 ml of 1:1000 dilution of either a yeast ferment or sterile fruit juice control. Experiments were run for 30 minutes in the absence of light to ensure that any choice made was done solely on olfactory cues (Palanca *et al.,* 2013; Buser *et al.,* 2014; Günther *et al.,* 2015; Günther *et al.,* 2019). Where combinations of yeasts were tested, yeasts were fermented separately and then combined in in equal proportions. T-maze position was randomised in regard to their orientation, in addition to treatments being reversed, to account for any directional bias in the room.

Flies were anaesthetised using CO_2 for a maximum of 10 minutes to separate out the number of females required and then starved for 24 ± 1 hours prior to the experiment start. For *D. suzukii* flies were starved for 17 ± 1 hours then anaesthetised and sexed. Flies were again anaesthetised briefly using CO_2 before being inserted in the centre of the T-Maze apparatus (Figure 1). Between 60-80 mated adult *D. suzukii* females between 3-12 days old were added to each T-maze. Each replicate run consisted of one replicate per yeast for all treatments; this was repeated six times. Thus, ensuring that each cohort of flies, bred from the original populations, was subjected to all treatments. Ensuring that the physiological state of each cohort of *Drosophila* was similar, to reduce variation between yeasts tested.

After each replicate run, T-mazes were placed in a freezer to euthanise the flies prior to counting. To determine attractiveness of each yeast species an Attraction Index (AI) was calculated (AI = the total number of flies in the arm corresponding to yeast ferments subtracted from the number of flies in the arm corresponding to the sterile strawberry juice, divided by the total number of flies making a choice) (Palanca *et al.,* 2013; Buser *et al.,* 2014;

Günther *et al.,* 2015). After completion of the experiment the sliding doors of the T-maze were closed (Figure 1). Any flies that left the central compartment, passed either sliding door thus occupying either arm, were deemed to have made a choice (Palanca *et al.,* 2013; Buser *et al.,* 2014; Günther *et al.,* 2015). The data was analysed using the binomial distribution to determine whether the choices made by the flies differed significantly from a random distribution (Palanca *et al.,* 2013; Buser *et al.,* 2014; Günther *et al.,* 2015).

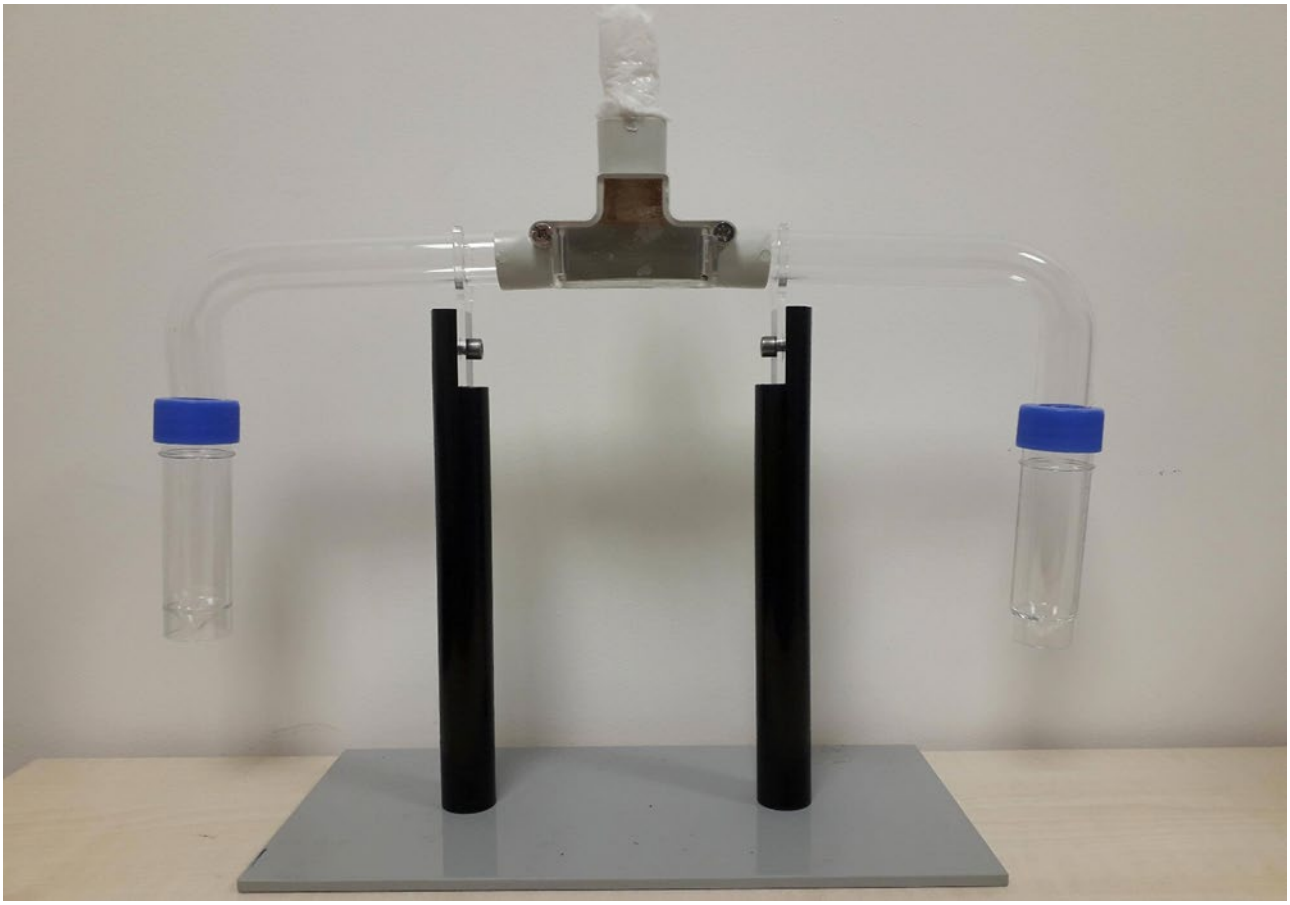

Figure 1: Set up of T-maze apparatus. 10 ml of 1:1000 dilution of yeast ferment or sterile fruit juice for a control was placed in the vials attached to the T-maze arms, a piece of circular mesh was placed in between the top of the vial and the end of the T-maze arms. This prevented the *Drosophila* individuals interacting with the ferments/fruit juice, whilst simultaneously allowing diffusion of odours from the different treatment throughout the T-maze. For all experiments using *D. suzukii,* damp blue absorbent paper was included in the centre of the T-maze to increase humidity. *Drosophila* individuals were placed in the T-maze after being anaesthetised. A cotton wool plug prevented any flies escaping. The doors of the T-maze were opened in the absence of light, so as to prevent the flies making a choice based on visual cues.

Field trials

Field experiments were conducted on a commercial fruit farm in Kent, UK. Drososan field traps (Koppert Biological Systems) were deployed with a 200 ml drowning solution of either single, or combinations of yeast fermented in strawberry juice. Where combinations of yeasts were required, equal volumes of singly fermented yeast were mixed to produce the 200 ml. In each trial, three control treatments were included; sterile strawberry juice, distilled water (negative control) and commercially available Gasser lure (RIGA) (positive control). Triton x 100 (Sigma-Aldrich, concentration of 0.005%) was added to all drowning solutions, regardless of treatment, to reduce surface tension. Traps were arranged in a randomised block design where one trap from each treatment was present in a random order per block. For tests 1 and 2, traps were situated in a native hedgerow approximately 3 m apart and 1 m from the ground adjacent to raspberries canes. For test three, traps were sited in a deciduous, bramble woodland, adjacent to the hedgerow close to the same raspberry canes. Traps were placed 7-8 m apart and 1 m from the ground. After 72 hours trap contents were filtered through muslin and numbers of male, female and other *Drosophila* were counted in the laboratory.

Microbes on fruit

Fruit samples were collected from four different ripening stages of strawberries, raspberries, blueberries and cherries (Figure 2). Samples were collected from three different locations in Kent. Four stages from all fruit cherry, blueberry, raspberry and strawberry were collected and processed (Figure 2). Ten fruit, 20 for blueberries, were aseptically removed from plants with as much of the stalks or calyx of the fruit being removed as possible without damaging the fruit epicarp. Six replicates of fruit species at each stage were used. After collection, the fruit was surface-washed with autoclaved water to remove microbes. The water used to wash the fruit was collected in 50 ml falcon tubes and centrifuged for 30 mins at 4,500rpm, whereupon the supernatant was discarded leaving approximately 2 ml in the tube. The pellet was then re-suspended in the remaining water and separated. 1 ml was added to 2 ml safe-lock tube and centrifuged for 30 mins at 13,000rpm whereupon the supernatant was discarded and the pellet frozen at -80°C for DNA extraction. Approximately 1 ml was mixed with equal parts 30% glycerol to create a 15% solution and frozen at -80°C for cultural-based analysis. 100 µL was added to 500 µL of YPD media and incubated at 30°C for 3 days. Cells were collected by centrifuging for 10 mins at 13,000, the supernatant was removed and the collected cells were re-suspended in 15% glycerol and stored at -80°C.

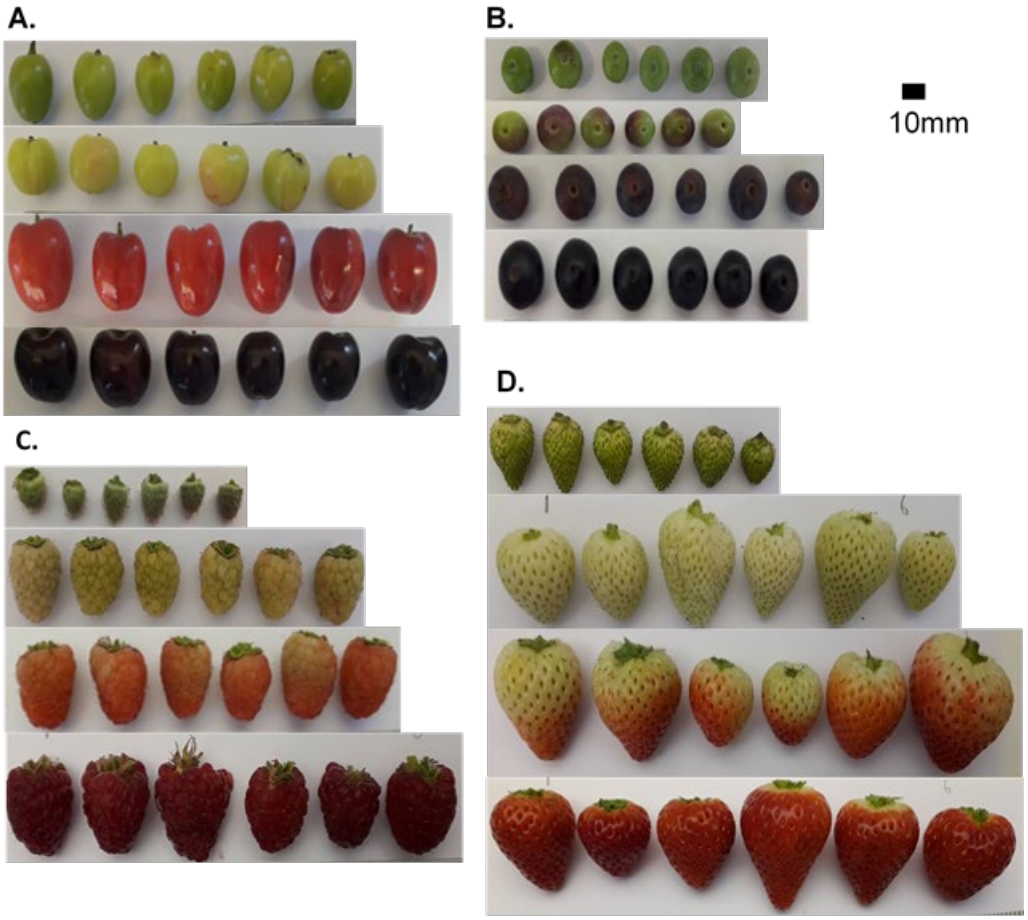

Figure 2: Four stages of fruit sampled and processed. A. green, white/pink, red and harvest (top to bottom) of cherry. B. green, green/purple, purple and harvest (top to bottom) of blueberry. C. green, white, pink and harvest (top to bottom) of raspberry, D. green, white and red/white (pink) and harvest (top to bottom) of strawberry.

DNA extraction

The pellets derived from samples were split into equal parts. Approximately 250 cells of a known benchmark fungal species, *Plectosphaerella cucumerina*, was added to one half of each sample in an attempt to quantify the absolute abundance of species. DNA was extracted using the DNeasy Blood and Tissue kit (QIAGEN) following the manufacturer's instructions but with a additional bead beating step, before incubation, were pellets were resuspended in 750 µL ATL buffer and moved to a bead tube containing 1g of glass beads at a 1:1 ratio of <106 µm and 0.5mm size beads (Sigma-Aldridge). Placed in a bead beater (Bead Ruptor 12, Omni international INC) a set on max speed for 30s, this was repeated 5 times.

Amplification

PCR reactions were set up using 15 µl Kapa 2x master mix (Kapa Biosystems), 6 µl of 1x ITS primer mix, 7 µl sterile water and 2µl template DNA. Each reaction included a negative control (2µl autoclave water) and a positive control (*S. cerevisiae* DNA). The PCR cycle parameters were 95°C for 3mins, 29 cycles of 98°C for 20 seconds 64°C for 20 seconds 72°C for 40 seconds, followed by a final extension time at 72°C for 5mins. The PCR product was electrophoresed on a 2% agarose gel containing 10 µl SYBR safe dye™ (Invitrogen) per 100ml TAG buffer. The gel was electrophoresed for 25mins at 120V, after which the size of the PCR product was ascertained by comparison to a DNA size ladder. PCR amplicons were sequenced on Illumina MiSeq instruments with a 300PE metric by Eurofins genomics.

Statistical analysis

Yeast attractiveness was analysed by ANOVA and binomial logistic regression, where treatment and Drosophila species were the main factors in the logistic regression model, and significance was assayed using ANOVA following model simplification by removal of the interaction between the two factors from the null model (Crawley, 2005). The attraction of individual yeast was analysed using the binomial distribution to determine whether the choices made by the flies differed significantly from a random choice distribution (Palanca *et al.,* 2013; Buser *et al.,* 2014; Günther *et al.,* 2015; Günther *et al.,* 2019). Permutation analysis was used to test whether yeasts interacted linearly or non-linearly in terms of attraction when combined into communities. The predicted AI based on an additive (linear) model was compared to the observed AI of yeast combinations. Predicted AI values for yeast combinations under an additive model were created by randomly selecting individual AI values from the replicates of each of the corresponding single yeast experiments for each of the constituent yeasts in the combinations and calculating the mean AI from these. This procedure was then repeated 1,000 times for each combination to generate a null/additive distribution of community AI values for each of the yeast combinations employed. The experimentally observed AI values from yeast combinations was then compared to this null distribution and used to calculate the probability the observed yeast combination AI was due to an additive interaction between constituents' yeasts in the communities. Additionally, a linear model was also used to test whether the preferences for the communities of yeast differed from that predicted based on a linear mixture of the preferences of the individual yeasts ($y = X\beta + \varepsilon$).

Field trap capture data was analysed using a linear regression with Poisson distribution. For total *D. suzukii*, interaction terms between the fixed effects of treatment and sex were

included, and block was included as a random effect. Significance was tested using ANOVA following model simplification by removal of interaction between treatment and sex from the null model. If there was a significant interaction with sex, *D. suzukii* males and females were analysed separately.

OTUs (operational taxonomic unit) were derived from DNA sequence reads by comparing all sequences to one another and pooling into OTUs of >97% similarity. Since this level of sequence homology approximates differences between species, from herein OTUs are referred to as species. A 2-way ANOVA was used to evaluate the effect of main factors on the number of OTUs. Both a 2-way Permanova binary Jaccard and standard Jaccard we used to determine if there was an effect of OTUs presence and relative abundance of OTUs respectively again both analyses tested if there was a significant effect of fruit type and ripening stage as well as an interaction between them.

All statistical analyses were carried out in in R studio version 1.1.453 (R Studio Team, 2015). Llme4 stats package was used for the binomial logistic regression and linear regression with Poisson distribution, both Emmeans and Hmisc stats packages were used to generate confidence intervals. Vegan stats package was used for PERMANOVA analysis.

Results

In the second year of this project, at least some work has been completed to contribute to testing H1, H2, H3 and H4; results are presented here.

H1-There is no significant difference between the attractiveness of different yeast species to *D. suzukii.*
And,
H2-There is no significant difference between the attractiveness of different *H. uvarum* strains to *D. suzukii*.

There was a significant interaction between yeast type and sex for numbers of *D. suzukii* captured, and this explained a significant amount of deviance in the trap data (Δdev=44, df=7, p<0.001). We therefore analysed male and female data separately. There was a significant difference between yeast type for numbers of male and female *D. suzukii* and other *Drosophila* caught (Δdev=4568, df=7, p<0.001, Δdev=1874, df=7, p<0.001 and Δdev=1118, df=7, p<0.001 respectively). Confidence intervals revealed all traps had significantly greater numbers of male and female *D. suzukii* (and other *Drosophila*) than the sterile water negative control. Overall the commercial Gasser-lure bait was the most attractive treatment attracting significantly more individuals than other treatments for both male and female *D. suzukii*. For other *Drosophila,* however, Gasser-lure was significantly more attractive than all treatments apart from *H. uvarum* (201). Three of the yeast treatments were more attractive than sterile strawberry juice: *H. uvarum* (201), *H. uvarum* (11-382) and 190 for male and female *D. suzukii* and other *Drosophila*. *H. uvarum* (201) was significantly more attractive than both *H. uvarum* (11-382) and 190 (Figure 3), and *H. uvarum* (11-382) and 190 were equally as attractive as each other and both significantly more attractive than 218 and *S. cerevisiae* which were equally as attractive as each other and not different in attraction from the sterile fruit control (Figure 3).

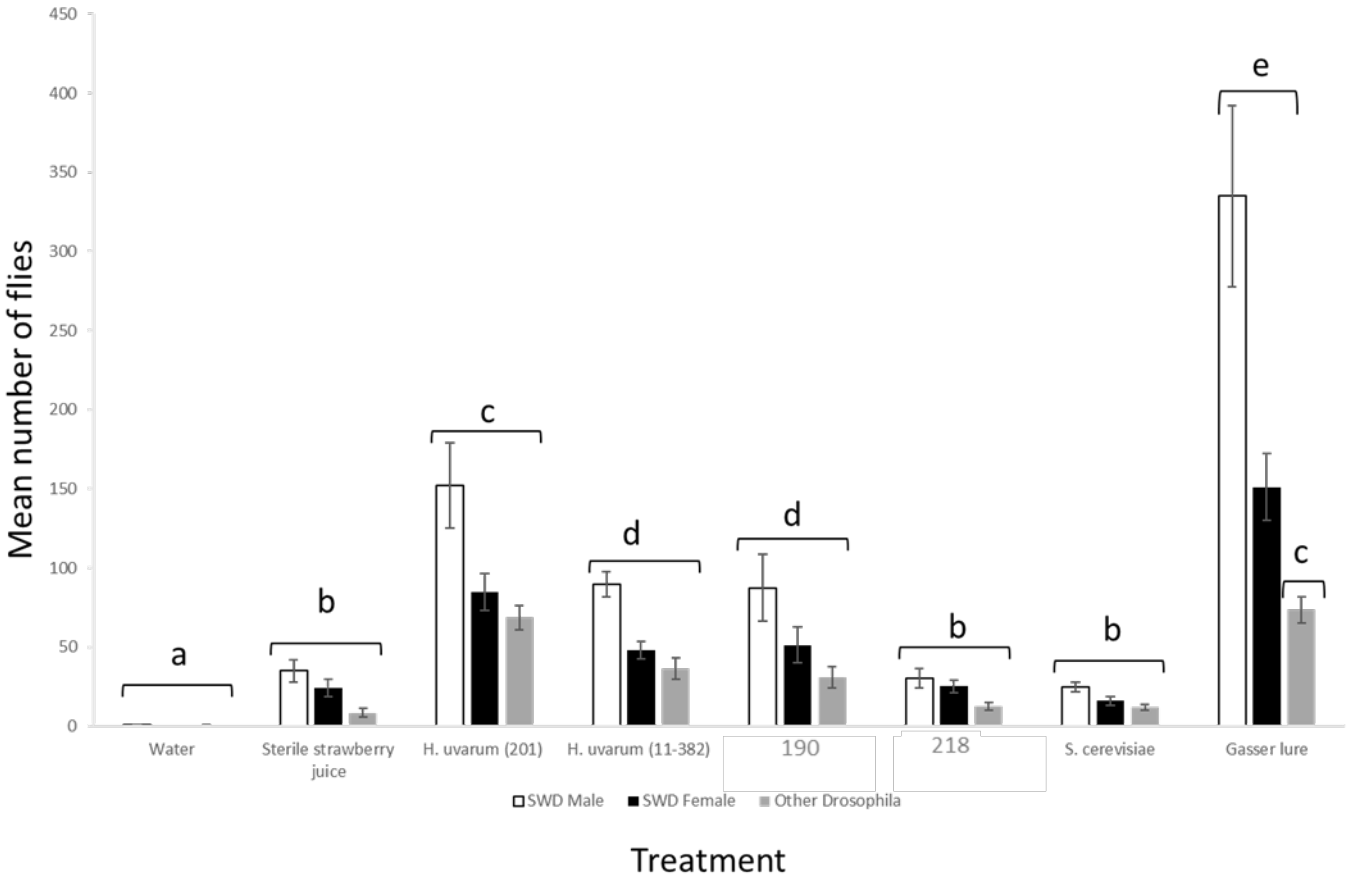

Figure 3: Mean numbers (± SE) of *D. suzukii* and other *Drosophila* attracted to five individual yeasts, four different species (*H. uvarum,* 190, 218 and *S. cerevisiae*) and two strains of *Hanseniaspora uvarum* (201 and 11-382) fermented in strawberry juice in comparison to sterile strawberry juice, water and commercial Gasser lure (N=6). Attraction is measured as the total number of flies caught over a 72 hour period. Lower case letters above bars shows statistical differences for non-overlapping confidence intervals, calculated from Estimated Marginal Means (95%).

H3- The effect of combining yeast on the attraction of *D. suzukii* will follow an additive (linear) model i.e. there will be no interaction effect or synergistic effect of combining yeast on attraction.

Two-way choice tests

There was a significant difference in attractiveness between yeast combinations (following model simplification, by removing treatment, deviance = -39.16, df=-12, p>0.001). Individual

binomial analyses reveal four yeast combinations to be significantly more attractive than sterile strawberry juice: 190+201 (AI=0.33, P<0.001), 201+164 (AI=0.23, P=0.0026), 190+218+201 (AI=0.19, P=0.010) and 190+218 (AI=0.18, P=0.0096). *H. uvarum* (201) alone was significantly attractive (AI=0.15, P=0.027), and the positive control Gasser lure was also attractive (AI=0.28, P<0.001). *D. suzukii* was indifferent to seven yeast combinations compared to sterile strawberry juice (Figure 4). We next tested whether there was any evidence that yeast volatiles combined into combinations interacted in a non-linear manner to affect the degree to which *D. suzukii* are attracted. Permutation analyses reveals an interaction between yeasts in two of the combinations: 190+218+201+164+98-3 and all yeasts (P=0.012 and P=0.006 respectively; Figure 4). For these combinations the observed AI values were significantly lower compared to predicted values assuming an additive interaction of the AIs from the constituent component yeasts in the combinations. This indicates there may be some types of yeast combinations that produce a combined volatile profile that is repulsive to *D. suzukii*. Analysis with a linear model also revealed that 190+218+201 +164+ 98-3 (P=0.0057) differed significantly from predicted attractiveness assuming a linear model of attraction, but the all-yeast combination did not (P=0.072).

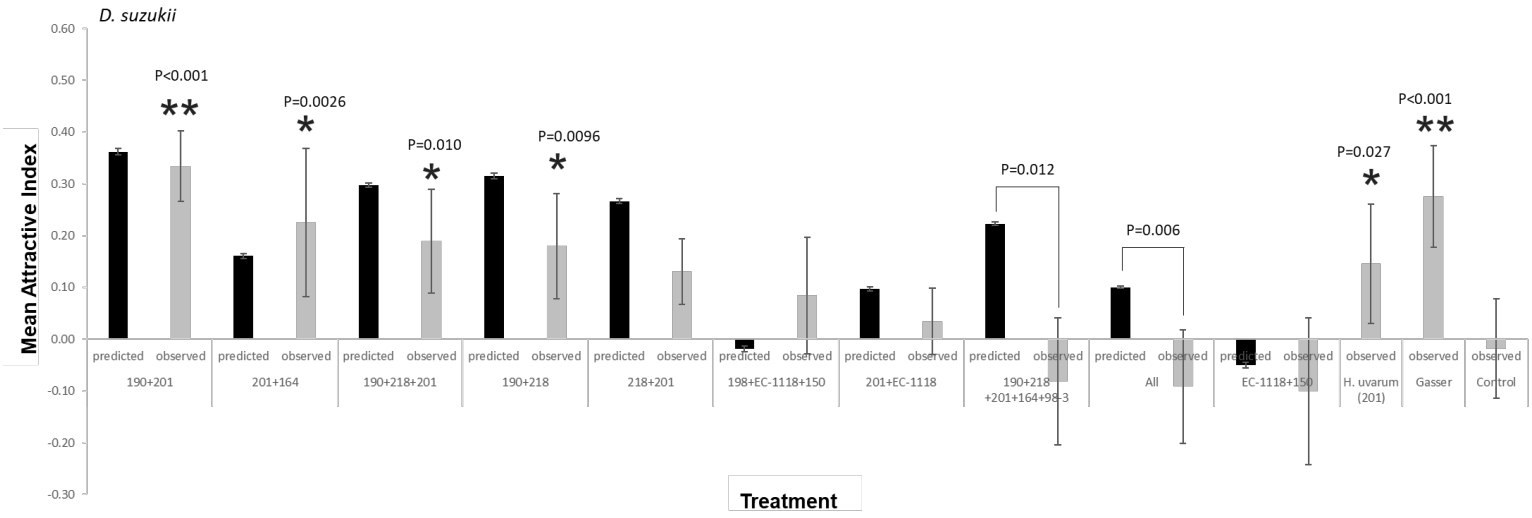

Figure 4: Mean ± standard error of the mean Attraction Index (AI) of *D. suzukii* to ten yeast combinations, fermented individually in strawberry juice then mixed in equal proportions. *H. uvarum* (201), sterile fruit and commercial Gasser lure act as controls. N=6 all treatments except Gasser, Hu (201), All, 190+218+201+164+ 98-3 and EC-1118+198+150 and control (N=7). *D. suzukii* had no significant preference for either side in the sterile fruit juice control (AI=-0.02, P=0.36). Grey bars represent observed AI and black bars the mean predicted AI, calculated from 10,000 permuted individual AI values. Asterisks above bars represent any significant attraction.

Field trials

Overall, there was a significant interaction between treatment and sex and this explained a significant amount of the deviance (Δdev=65, df=8, p<0.001 and Δdev=247, df=8, p<0.001, first and second sites respectively), and so male and female *D. suzukii* were analysed separately. There was significant variance in attraction to different yeast treatments for male and female *D. suzukii* and other *Drosophila* (male: Δdev=1323, df=8, p<0.001, Δdev=1299, df=8, p<0.001 and female: Δdev=1437, df=8, p<0.001 first site, and male: Δdev=9366, df=8, p<0.001, Δdev=9661, df=8, p<0.001 and female: Δdev=2613, df=8, p<0.001 second site). The confidence intervals for male and female *D. suzukii* and other *Drosophila* revealed all treatments were more attractive that the negative water control for both sites and that Gasser-lure was the most attractive. *H. uvarum* (201) was significantly more attractive than sterile strawberry juice for male *D. suzukii* at the first site only. Individual analysis of male *D. suzukii* in traps from the first site reveals two yeast combinations were more attractive than sterile fruit: 190+218+201 and 190+201, and only one yeast combination was more attractive than sterile fruit for female *D. suzukii* was 190+201. Four of the yeast treatments proved to be more attractive than sterile strawberry juice for other *Drosophila*; *H. uvarum* (201), 190+201, 218+201 and 201+EC-1118 (Figure 5a; first site). For the second site only, one yeast community was more attractive than sterile fruit for male *D. suzukii:* 190+201. Sterile strawberry juice, *H. uvarum* (201), 190+218+201, 190+218 and 190+201 were more attractive than 218+201. 190+218 and 190+201 were also more attractive than 201+EC-1118. For female *D. suzukii,* two combinations were more attractive than sterile strawberry juice; 190+218 and 190+201, and these combinations were significantly more attractive than 201+EC-1118 and 218+201. For other *Drosophila* the 'all yeast' treatments were more attractive than sterile strawberry juice, with *H. uvarum* (201) and 190+201 also being more attractive than 190+218 and 218+201 (Figure 5b).

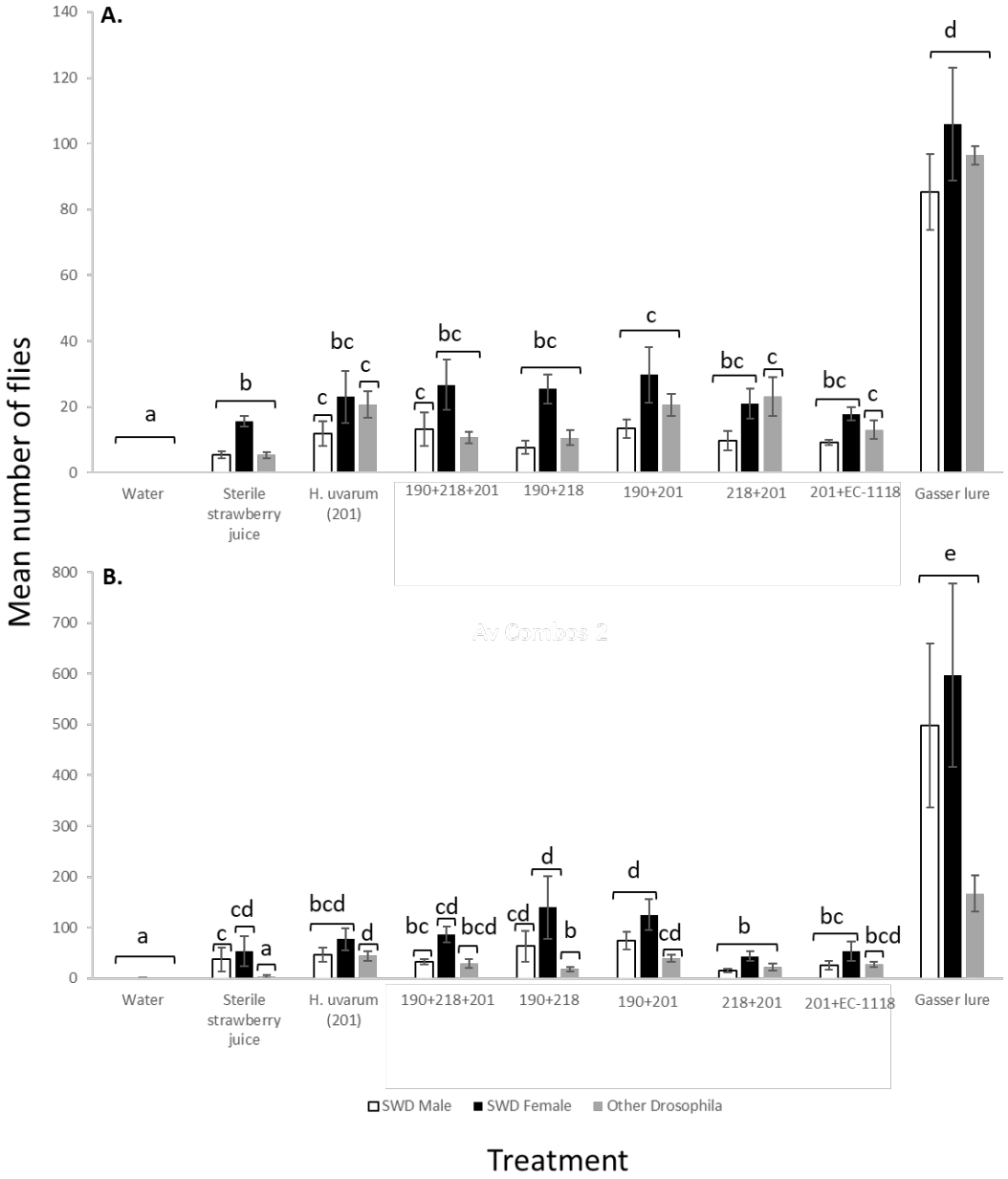

Figure 5: Mean numbers (± SE) of *D. suzukii* and other *Drosophila* attracted to five combinations of yeast species (190+218+201, 190+218, 190+201, 218+201, 201+EC-1118) fermented separately in strawberry juice and then combined in equal proportions. *H. uvarum* (201), sterile strawberry juice, water and commercial Gasser lure serve as controls. N=7 except 190+218 N=6 at the second site. A. first site carried out in November 2018 at the same native hedgerow at the commercial fruit producer in Kent, UK as the single yeast field test; B. carried out December 2018 in a deciduous bramble woodland, adjacent to the site used in A. Attraction was measured as the total number of flies caught over a 72 hour period. Lower case letters above bars shows statistical differences (with in group) for none overlapping confidence intervals, calculated from Estimated Marginal Means (95%).

H4-There are no significant differences in the yeast communities on ripening fruit, regardless of fruit type or stage of ripening.

There was a significant effect of fruit type and ripening stage on the number of species (ANOVA, $F_{(1,88)} = 42.30$, $p < 0.001$ and $F_{(3,88)} = 5.50$, $p = 0.0017$ respectively), with an average of 117 species for cherry and 78 for blueberry and an average increase of 30 species from 1st to 4th ripening stage. However, there was no significant interaction between fruit and ripening stage. For blueberries, there was no significant effect of ripening stage on number of species ($F_{(3,44)} = 1.83$; $P = 0.16$), but there was a significant effect of ripening stage for cherry ($F_{(3,44)} = 11.34$, $p = 0.0085$). Post-hoc tests revealed that there were significant differences between species numbers as cherry ripens, with an average of 40 more species at stage 4 compared to stage 1 and 33 to stage 2 (Figure 6).

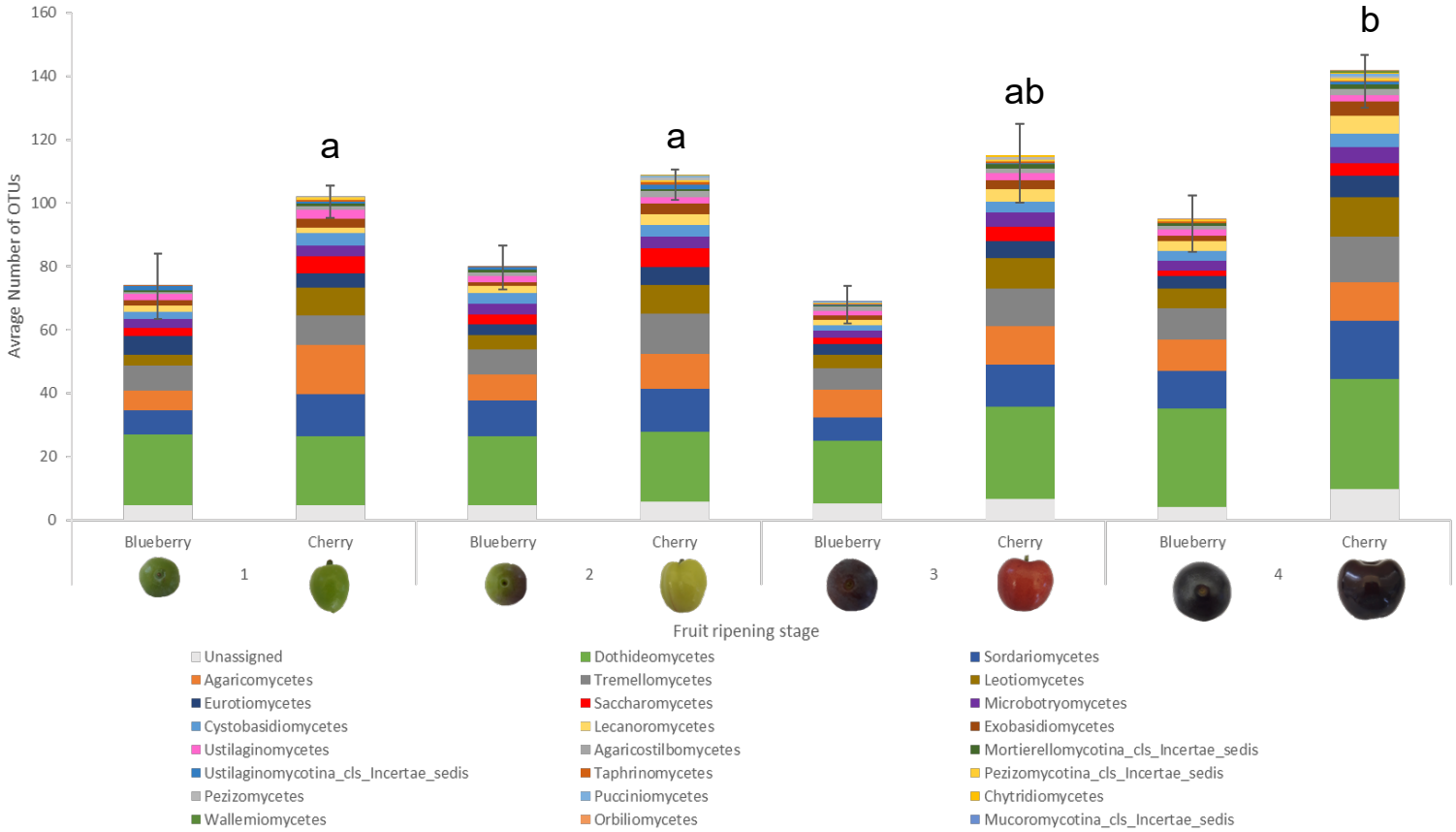

Figure 6: Average number of OTUs detected on four ripening stages of blueberry and cherry fruit. Colour portion of each bar represents percent of each taxonomic class of the OTUs at the corresponding fruit stage. Bars connected by the same letter do not significantly differ (test, alpha = 0.05).

Differences in types of species were evaluated with 2-way PERMANOVA on binary Jaccard distance matrices and revealed there was a significant effect of both fruit and ripening stage (R^2=0.099, p>0.001 and R^2=0.025, p>0.001 respectively), and significant interaction between fruit and ripening stage (R^2=0.020, p>0.001). Differences in the abundances of species were evaluated using a 2-way PERMANOVA on abundance based Jaccard distances and revealed that fruit type and ripening stage also had significant effects and the presence of a significant interaction between these factors (R^2=0.42 p>0.001, R^2=0.034 p>0.001 and R^2=0.043 p>0.001 respectively). There was a significant effect of ripening stage for both blueberry and cherry for both the types and abundances of species across ripening stage (blueberry: R^2=0.043, p>0.001, R^2=0.17, p>0.001; cherry: R^2=0.060, p>0.001, R^2=0.061, p=0.0066). Relationships between communities from cherries and blueberries and four ripening stages are shown in (Figure 7).

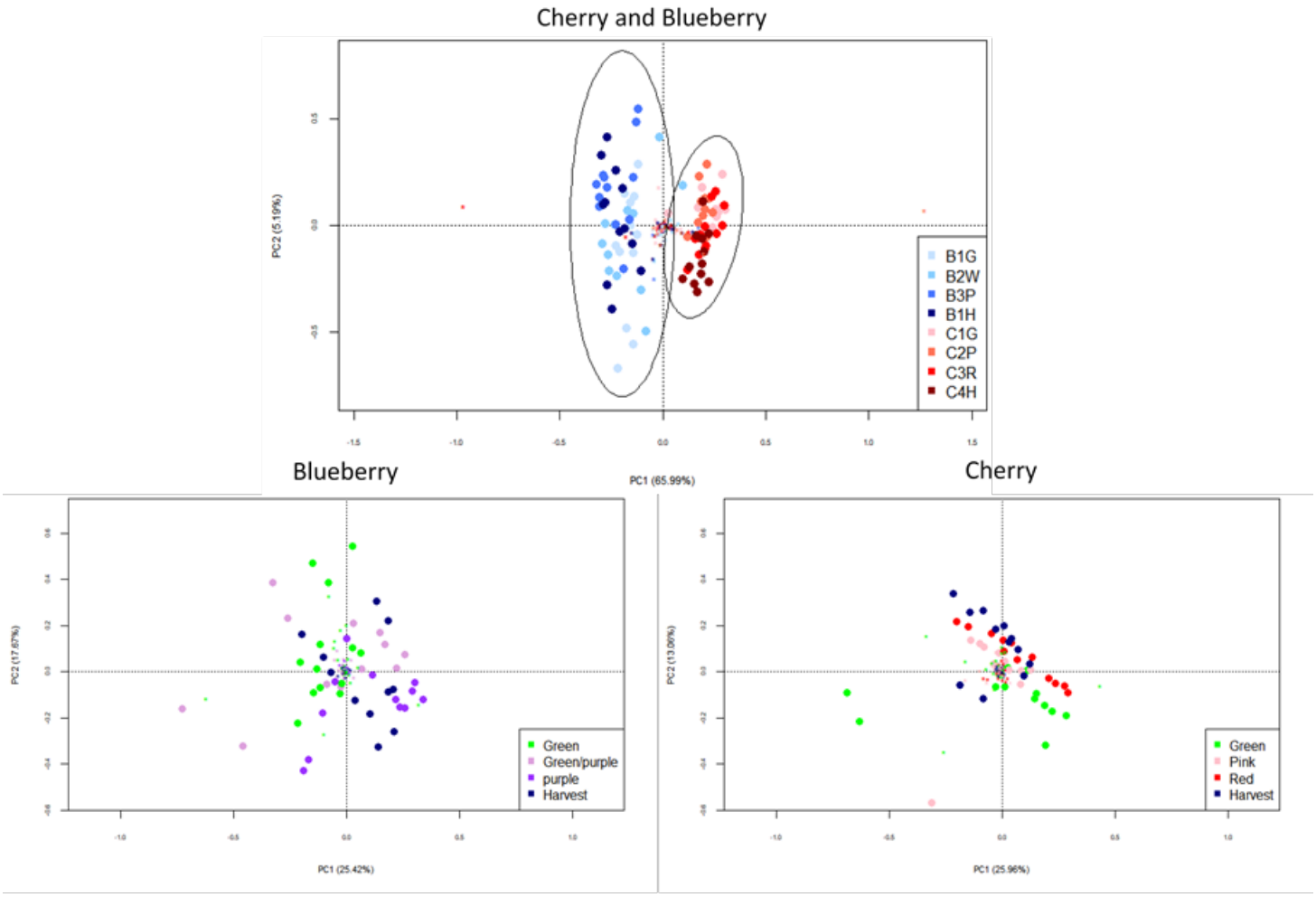

Figure 7: Principal component analysis (PCA) of abundances of fungal species on four different ripening stages of blueberry and cherry.

Discussion

The attractiveness of different yeast species to different *Drosophila* was shown to vary (Figure 3); this is in line with the findings from last year's report and with previous studies (Palanca *et al.,* 2013; Buser *et al.,* 2014; Günther *et al.,* 2015; Scheidler *et al.*, 2015). In the field, *Drosophila suzukii* was significantly attracted to three yeasts: the two *H. uvarum* strains and 190; and *S. cerevisiae* was no more attractive than sterile strawberry juice (Figure 3). However, unlike the laboratory findings, 218 was not significantly attractive in the field. Overall this suggests that 2-way choice test with T-mazes are a good proxy for attraction in the field, but highlight the need for field testing to confirm attraction.

Scheidler *et al.* (2015) found that *H. uvarum* and *S. cerevisiae* were both significantly attractive to *D. suzukii.* In contrast, we have again reported that *D. suzukii* is indifferent to *S. cerevisiae* (EC-1118). We assayed the attractiveness of yeast species fermented in strawberry juice whereas Scheidler *et al.* (2015) used culture media, Potato Dextrose Broth, which could account for the differences. Also, different strains of *S. cerevisiae* were used which could also account for the difference in attractiveness (Scheidler *et al.*, 2015) as it has been shown that different *S. cerevisiae* genotypes vary in their attractiveness to *D. simulans* (Buser *et al.,* 2015) and *D. melanogaster* (Palancar *et al.,* 2013). The attractiveness of *H. uvarum* strains seems to vary, with there being a significant effect of attraction between the two strains tested in the field (Figure 3).

To date, only single yeasts have been tested for attraction to *Drosophila* (e.g. Palanca et al. 2013; Buser et al. 2014; Scheidler et al. 2015). We combined singly fermented yeast to create combinations and found that 4 of the 10 combinations tested were significantly attractive to *D. suzukii* Including 190+218+201, 190+218, 190+201, 201+164 (Figure 4). In the field, three of the combinations tested caught significantly more flies than sterile strawberry juice for both female and male *D. suzukii* 190+201 (across both field assays), 190+218 for female (only second) and 190+218+201 for males (only first)(Figure 5). Additionally, there seemed to be some evidence that, at least for some combinations, attraction does not follow a linear model as two of the combinations 190+218+201+164+98-3 and 'all yeast' differed significantly from expected additive values of attractiveness (Figure 4).

Fruit surfaces are home to complex microbial communities which are often dynamic. For blueberries we found that there was no significant effect of ripening stage species number. However, for cherry there was a significant effect of ripening stage on species number, with

significant differences between species numbers at cherry ripening stages 1, 2 and 4 (Figure 6). In addition, there was a significant effect of fruit type and ripening stage for both the types and relative abundance of species. Microbial communities are known to be affected by a range of factors including fruit species, ripening stage, geographic location and damage sustained by fruit (Leff and Fierer, 2013; Barata *et al.,* 2012; Taylor *et al.,* 2014 Vepštaitė-Monstavičė *et al.,* 2018).

Currently, existing baits used for attracting *D. suzukii* are both unselective and not competitive with ripening fruit (Mori *et al.,* 2016). This makes sorting trap captures time-consuming and difficult as *Drosophila* are often hard to identify; *D. suzukii* females can be mistakenly identified for other *Drosophila* species without the aid of a microscope. As yeast species are differentially attractive to *Drosophila* species (Palanca *et al.,* 2013; Scheidler *et al.,* 2015; Günther *et al.,* 2019) yeast-based baits could potentially produce a highly attractive and selective bait, potentially reducing non-target trap captures making it easier to detect *D. suzukii* and sort through trap captures. As *D. suzukii* is attracted to ripening fruit, unlike most other species of *Drosophila*, this potentially provides an opportunity to exploit yeast species or communities present on ripening fruit to find an attractive and selective yeast attractant that could be used in control strategies for this pest.

Conclusions

Yeasts vary in their attraction to *Drosophila* species and previous work in 2017/18 identified four candidate yeast species attractive to *D. suzukii*: *H. uvarum*, yeast coded 218, 164, and 190. Of these yeasts three were also attractive in the field, yeast coded 190, and two stains of *H. uvarum*. Four combinations proved to be significantly attractive in the laboratory 201+164, 190+201, 190+218+201 and 190+218, but in the field only the latter three combinations attracted *D. suzukii*. There seems to be a good correlation between attraction in the laboratory and field, suggesting that 2-way choice test with T-mazes are a good proxy for *D. suzukii* attraction in the field, but also highlighting the need for field testing to confirm attraction. Currently, none of the yeast-based attractants tested in the field proved significantly more attractive than commercial Gasser lure. It is worth noting that these field-based trials were conducted between late October and early December.

Preliminary analyses have revealed that for cherries there was a significant effect of ripening stage on species presence, but for blueberries this was not the case. However, for both presence/absence and relative abundance of species there was a significant effect of fruit

type and ripening stage as well as an interaction between them showing that fungal communities vary between both fruit type and ripening stage (Figure 6, 7).

Knowledge and Technology Transfer

ABB Crop Protection in Southern Britain 12-13th November 2018.

PGR symposium at University of Lincoln on in 10th October 2018 (Poster).

AHDB Studentship Conference 2017(flash talk), 2018 (Poster).

ABB Advances in IPM 2018: Making it Work for the Farmer 12-13 December 2018 (Poster).

AHDB/NIAB EMR Association soft fruit day on the 21st November 2018 (talk and poster,winning poster in poster competition).

SF145a Field meeting 4.10.19 (talk)

Publication

Günther, C.S., Knight, S.J., Jones, R. and Goddard, M.R., 2019. Are Drosophila preferences for yeasts stable or contextual? *Ecology and evolution*, *9*(14), pp.8075-8086.

Upcoming

Have been accepted to present a talk at the ABB Advances in Biocontrol & IPM 2019: Addressing the innovation crisis. November 2019.

AHDB/NIAB EMR Association soft fruit day November 2019 (Poster).

AHDB Studentship Conference (Talk).

Plan on applying to present a talk at the International Congress of Entomology conference in Helsinki 2020.

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