Project title:	Enhancing control of the soft- and stone- fruit pest *Drosophila suzukii* (Spotted Wing *Drosophila*) by exploiting its activity patterns in the field.
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Project leader:	Michelle Fountain, NIAB EMR, KENT. Herman Wijnen, University of Southampton
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The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

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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CONTENTS

GROWER SUMMARY

Headline

- New knowledge gained about the daily activity of spotted wing drosophila

Background and expected deliverables

Daily behavioural and physiological rhythms, such as activity and sleep, are exhibited by a wide range of organisms as a result of the interaction between environmental cycles and an internal timekeeping mechanism known as the circadian clock (Allada and Chung, 2010, Bollinger and Schibler, 2014). The clock can be regulated by environmental cues including temperature and daylight and enables synchronicity to dynamic daily conditions (Dubowy and Sehgal, 2017, Dubruille and Emery, 2008).

D. suzukii is an invasive species that damages ripening fruit, unlike other Drosophila species which only feed on overripe or spoiled fruit (Rota-Stabelli et al., 2013). *D. suzukii* was found for the first time in the UK in an area of wild blackberry in Kent in August 2012 (Harris and Shaw, 2014) and is now a serious pest in protected and outdoor cherry, strawberry, raspberry, blackberry and blueberry. Female *D. suzukii* are able to insert eggs into the skin of ripening fruits with a serrated ovipositor. Once the eggs hatch, the larval stages consume the fruit from within causing the fruit to collapse rendering fruit unmarketable.

Further insight into the daily behavioural and physiological rhythms of *D. suzukii*, as determined by its internal circadian clock and environmental cues, may help to predict the times of day when *D. suzukii* poses the greatest threat to crops and when they would be most vulnerable to control measures. The research that would be invaluable to British soft- and stone-fruit growers would include exploitation of the behaviour and physiology of *D. suzukii* to enable more effective control within the UK growing season.

In recent years there has been a surge in the numbers of studies (in the USA and Europe) on the behaviour and control of *D. suzukii*. On-going studies in the UK (e.g. AHDB project SF/TF 145a) are demonstrating peaks of seasonal activity. However, little is known about the daily and seasonal rhythmicity in the behaviour and physiology of *D. suzukii*. Current work on behavioural rhythms of this pest has been done in other countries under different environmental conditions, which, due to the nature of the clock, may result in different behavioural outputs.

Hamby et al. (2013) described diurnal fluctuations in *D. suzukii* locomotor activity under laboratory conditions mimicking summer and winter days and Evans et al. (2017)

investigated the oviposition rhythms of *D. suzukii* in outdoor field studies. However, both these examples were performed under temperature and light cycles that we would not experience in the UK and used unrealistic social grouping to reach their conclusions. More expansive studies of clock-controlled daily rhythms have been done on the *D. suzukii* sister species, *D. melanogaster*. The latter exhibits not only circadian locomotor behaviour, gene expression and metabolism, but also daily clock-controlled oscillations in processes such as feeding, egg laying and eclosion (adult emerging from pupae) (Xu et al., 2008). Behavioural patterns and rhythms have been observed in *D. suzukii* in constant conditions and so the influence of environmental and social conditions have not been evaluated (Lin et al., 2014).

To tailor control methods to specific behavioural events, a greater understanding of what drives or influences these behaviours is needed. In this project, rhythms in activity (locomotion), oviposition and susceptibility to plant protection products is investigated. The parameters that influence or disrupt these physiological processes will also be explored to identify the correct methods, which can be used to predict 'wild type' behavioural patterns in an artificial setting. This will enable researchers to repeatedly run simulations in a controlled, laboratory environment and could then be used as a tool to predict patterns of key behaviours at specific times of the cropping year.

Aims and methods

The overall aim of this research was to investigate daily and seasonal rhythms of *D. suzukii* locomotion activity and oviposition and formulate recommendations for UK growers in regard to field detection, trapping and crop protection.

Locomotion activity was investigated using an electronic device that monitors movement of Drosophila under various environmental conditions in the laboratory. Individual males, individual females, groups of males, groups of females and mixed sex groups were monitored under different seasonal conditions to determine an average locomotion pattern. The effects of removing environmental cues on the locomotion activity were investigated to understand the mechanisms that drive the internal circadian clock. Locomotion behaviour was also observed under natural light and temperature conditions in a semi-field setting. These environmental conditions were recreated in the laboratory to confirm whether laboratory locomotion patterns would correlate with those collected under natural conditions in the field when under the same temperature and light cycles.

Oviposition patterns of wild *D. suzukii* populations were determined within a strategic cherry orchard in Kent. Traps were baited with fresh fruit and were changed every two hours from sunrise to sunset for three days. The emergence of the next generation was counted to identify peaks in egg laying. The environmental conditions collected during the field

oviposition trial were recreated in the laboratory and the oviposition pattern of laboratory strains recorded using the same method as in the field. Locomotion was also investigated under these environmental conditions to determine if there was a correlation between locomotion activity and oviposition.

To identify how to disrupt egg laying in *D. suzukii*, reproductive competition between *D. suzukii* and *D. melanogaster* was investigated. Each species was presented with a substrate either pre-inoculated with the opposing species' eggs or a blank substrate and the emergence of the next generation was counted.

To investigate the relationship between *D. suzukii* and UK approved plant protection products, laboratory based spray trials were performed. Sub-lethal doses of cyantraniliprole, lambda-cyhalothrin, pyrethrum and spinosad were directly applied to groups of *D. suzukii* via a bench top sprayer. Mortality, oviposition and the transgenerational effects of these doses were measured over time. In addition, the impact time of application was explored on mortality and oviposition by applying plant protection products at different time points.

Summary of the project and main conclusions

In the first year of this PhD study, it was demonstrated that the standard laboratory conditions for investigating circadian rhythms were not appropriate to predict behaviour in the field. Constant temperatures and 12:12 hour light: dark cycles produced very different locomotion patterns to fluctuating temperatures and seasonal day length. Locomotion patterns for individual flies differed from single sex and mixed sex groups. Locomotion activity was mainly driven by light and then influenced by temperature cycles. Within the second year of this PhD, a wider range of semi-field and laboratory conditions were investigated to see if laboratory strains would exhibit the same locomotion activity as those under the natural cycles. When these conditions were re-created in the laboratory, behavioural patterns of mixed sex groups and female groups correlated with those collected in the field. As this was successful, the results confirm that predictions of locomotion activity under natural conditions can be made from laboratory based assays if the correct environmental conditions are used.

Oviposition was investigated in August and October conditions and in both trials more eggs were laid in the day time than at night. In August, oviposition fell when temperatures exceeded 30°C and peak egg laying occurred in the mid-morning and evening when temperatures were between 25-29°C. In October, peak egg laying occurred at peak temperature each day. When the environmental patterns were recreated in the laboratory, laboratory strains displayed the same oviposition pattern as their wild counterparts. As with the locomotion activity, oviposition patterns of lab strains, when exposed to the recreated

environmental conditions, mirrored those collected in the field. This means that oviposition rhythms can also be predicted for specific times of the year if an average light and temperature cycle is known. The locomotion of mixed sex groups, when exposed to the oviposition environmental conditions, correlated with the patterns of oviposition indicating that some of the locomotion activity during the day is the result of females searching for oviposition sites.

In the reproductive competition assay, significantly fewer *D. suzukii* emerged from the *D. melanogaster* pre-inoculated substrate than the un-inoculated substrate. This reduction is caused by female oviposition choice, with fewer eggs being laid initially. However, there may also be some egg predation with *D. melanogaster* larvae feeding on *D. suzukii* eggs and/or larvae.

In most cases, the application of sub-lethal doses of a plant protection product resulted in significant reductions in survival and overall oviposition. However for cyantraniliprole (Exirel) more eggs were laid, overall, by females that were treated with 0.3% of the field rate (FR). There was also no significant reduction in survival of the offspring. For all of the PPP doses applied, there was a significant reduction in the number of eggs laid per female within 24 hours of PPP application. There were exceptions to this but typically they were <12% of the field rate. Over time, surviving females recovered from the sub-lethal doses and continued to lay eggs. One week after application, females that survived 100% field rate of spinosad (Tracer) laid the same number of eggs as untreated females.

There were also some transgenerational effects on offspring when parents were treated with lambda-cyhalothrin (Hallmark) and spinosad. There was a significant reduction in egg to pupa, and egg to adult survival 24 hrs after application with 12, 25 and 100% of the spinosad field rate and those treated with 6% of lambda-cyhalothrin. Forty-eight hours after application there was a significant reduction in pupa to adult survival when parents were treated with 6% of lambda-cyhalothrin field rate. There was also a significant reduction in survival from egg to adult 96 and 168 hrs after application with 6 and 100% field rate of spinosad.

The results from this assay highlight the importance of following a plant protection product labelling and using the field rate stated by the producer. In many cases, using lower than the recommended field rate resulted in reduced mortality and insignificant effects on oviposition of surviving females or offspring survival. Repeatedly exposing *D. suzukii* to sub-lethal doses of topically applied plant protection products could lead to insecticide resistance.

There was no impact of time of plant protection product application in either mortality or oviposition rates of *D. suzukii* to any of the four PPPs applied. Further work on this topic is needed to cover more PPP application time points.

Conclusions

- Daily activity (locomotion) rhythms of *Drosophila suzukii* are dictated by light primarily, and then by temperature, resulting in more general activity during daylight.
- Groups of *D. suzukii* display different locomotion patterns compared to individual *D. suzukii*, housed alone, and so groups should be used to make predictions about 'wild type' behavioural rhythms.
- Locomotion and oviposition rhythms under natural conditions can be predicted in the laboratory when the correct social conditions, in addition to realistic temperature and light profiles, are used.
- Oviposition occurs in the daytime even during short daylengths and mild night conditions.
- Oviposition reduces significantly when temperatures exceed 30°C.
- *D. suzukii* emergence is reduced when in competition with *D. melanogaster* for egg laying sites.
- Female oviposition rates recover, over time, if adults recover after being treated with sub-lethal and field rate doses of pyrethrum, lambda-cyhalothrin and spinosad.
- There are some transgenerational effects of plant protection product application on offspring development and survival.
- There is no impact of time of PPP application on *D. suzukii* susceptibility, of 4 orchard plant protection products in this study.

Financial benefits

According to Defra Horticultural Statistics, the value of UK produced cherries in 2017 was £23.6 million and without the availability of effective control methods, the total losses to the pest would not be far short of this figure as D. suzukii can give rise to almost 100% loss of a cherry crop. Raspberry, blackberry, blueberry and strawberry are also extremely susceptible and losses of 50% and more are not uncommon where effective control is not implemented. With the value of UK strawberry in 2017 estimated to total £283 million and raspberry estimated to be £128 million, then losses of half of these would amount to around £141.5 million and £64 million if uncontrolled.

The research in this project is filling gaps that are not being addressed in research programmes in other projects and other countries. It is essential for maintaining the viability and profitability of the UK's important soft and stone fruit industries. *D. suzukii* seriously threatens the sustainability of production of these crops in the UK. By understanding the Chrono-physiology of the pest we can provide growers with a better understanding of how to target the control of *D. suzukii*.

Action points for growers

- Use monitoring traps to monitor flying adults but take into consideration that trap catches will be low at temperatures below 10^{o}C and above 30^{o}C.
- Do not eradicate native *Drosophila* species from a crop as the oviposition competition may be restricting population expansion in ripened and waste fruit.
- It is essential to remove waste fruit from the crop frequently to reduce oviposition and feeding sites for *D. suzukii*.
- Always follow label rate recommendations for PPPs and rotate modes of action. Reducing the dose could result in resistance build up in D. suzukii.

SCIENCE SECTION

Introduction

Drosophila suzukii (Matsumura) is a relatively new invasive pest affecting British fruit production, being first identified in Kent in an area of wild blackberry in August 2012 (Harris and Shaw, 2014). Since its arrival in the UK it has caused commercial losses in cherry and some soft fruit crops, with some very small growers abandoning cherry orchards due to extensive fruit damage (pers. comms. anonymous). The exact cost of economic loss in unclear as some growers were not aware of the pest and attributed losses to other pest or pathogens. *D. suzukii* is threatening soft- and stone-fruit production as it is one of only two species of Drosophila that are able to lay eggs in intact, healthy and unripe fruit (Goodhue et al., 2011). Female *D. suzukii* have serrated oviscapts which are used to cut into ripe and ripening soft- and stone-fruit to insert eggs under the fruits epicarp (Kanzawa, 1935). The other species, *D. subpulchrella*, is not currently a significant threat to the global fruit market and is unable to lay eggs in thicker skinned fruits such as grapes (Takamori et al., 2006, Atallah et al., 2014).

The egg insertion hole made by *D. suzukii* exposes fruit to further attacks from pathogens and other insects that would not have been able to enter the undamaged fruit (Goodhue *et al*., 2011). Eggs hatch within the fruit and then develop through three larval instars (Walsh et al., 2011) which feed on the fruit flesh causing it to collapse and, in some cases, cause a melting appearance making fruit unmarketable. The larvae typically pupate in the fruit, but can leave the fruit to pupate before emerging as an adult. The cycle from egg to adult can be as rapid as 10 days in constant 30°C in laboratory conditions (Tochen et al., 2014). It is calculated that between 3 and 9 generations a year are realistic for the UK when estimated against the average seasonal temperature.

The Circadian Clock

Many organisms have an internal clock that controls behaviours in daily (circadian) and/or annual (circannual) cycles (Bollinger and Schibler, 2014). The circadian clock is a system that controls daily physiological behaviours including activity, feeding, sleeping and mating and is seen in organisms across all classifications. The clock regulates many functions which are triggered by external cues or zeitgebers (translation of German; ‘zeit’ meaning time and ‘geber’ meaning giver resulting in the literal translation of ‘time giver’). The functions are in response to daily environmental cycles (Hardin, 2005) such as light, which is the strongest (Schmal et al., 2015). By having a clock that is entrained to environmental

cues, individuals of the same species become synchronised in behaviours and processes such as courtship and reproduction, which then occur at the same time. This also means that organisms are able to synchronise with the environment, for example having offspring in summer when food is abundant and not in the middle of winter (Pittendrigh, 1960).

Traditional verses novel conditions to investigate circadian rhythms

One of the easier output behaviours of the clock to observe is activity or locomotion, and has been under investigation in *Drosophila melanogaster* for many years (Schlichting and Helfrich-Forster, 2015). *Drosophila* locomotion is measured in equipment known as *Drosophila* Activity Monitors, DAM, where individual flies are held in a glass tube (Figure 1). The tubes are held within a TriKinetics device and as the fly moves from one end to the other and breaks an infrared beam which is recorded as activity (Pfeiffenberger et al., 2010). These assays have been extensively utilised to understand how the removal of clock genes influences fly circadian rhythms. For this, flies are typically exposed to constant temperatures in either a Light:Dark (L:D), Light:Light (L:L) or Dark:Dark (D:D) regime. As behavioural patterns are regulated by the circadian clock, disruptions in rhythmicity are used to indicate the impact various factors have on an organisms' time keeping (Allada and Chung, 2010). As an example, by mutating *cyc*, a key circadian gene, flies become arrhythmic in locomotion activity when subjected to constant darkness, in comparison with a wild-type control (Rutila et al., 1998).

Figure 1. *Drosophila* Activity Monitor (DAM)used to record activity of individual Drosophila by passing through infer-red beam. The use of constant temperatures and either lights on or lights off monitoring has provided valuable insights in to the mechanisms

of the circadian clock. It is becoming more apparent that these artificial environmental conditions produce greatly variable behaviours to those that fluctuate through-out the day. In *Drosophila* variation in behaviours occur when ramping temperature cycles are used (gradually increasing or decreasing to mimic natural temperature cycles). Generally, under constant temperatures and a 12:12 L:D cycle, *D. melanogaster* display a morning and evening peak of activity and what is regarded as a 'siesta' during the middle of the light phase. When exposed to a ramping temperature cycle the afternoon siesta is replaced with a peak in activity (Menegazzi et al., 2012, Green et al., 2015).

Individual virgin flies are typically used within the DAM equipment, however, a recent study by Ferguson et al. (2015) discovered that virgin *D. melanogaster* females were recorded to exhibit similar activity patterns to that of mated males. The locomotion of virgin females differed greatly from that displayed by mated females as mated females displayed a peak of activity in place of the afternoon siesta and are generally more active throughout the day that the virgin females. Not only are mated flies rarely used in these assays, but very few researchers monitor locomotion patterns of groups of flies; either single or mix sex groups. Courtship and social interaction in *Drosophila* involve a large amount of movement and variety from species to species (Spieth, 1974). The ritual in *D. suzukii* includes male wing flashing and circling of the female and can take several minutes (Revadi et al., 2015). It is likely that the variation in locomotion activity between individual flies and mix sex groups is largely caused by courtship interaction; however, this is not investigated in the current literature.

Laboratory verses field

The ability to power recording equipment and protect it from weather conditions restricts where field based assays can be performed. For this reason there are many benefits to performing assays within a laboratory setting in comparision to the field including reproducability and practicallity (Nagy et al., 2018). However, there have been incidences where behaviour within a natural environment greatly differs from that observed within a laboratory. The golden hamster, *Mesocricetus auratus* (Waterhouse), when housed in the laboratory under 12:12 L:D at 22^{o}C performed 80% of activity at night leading researchers to believe they were nocturnal. However, in wild populations, females displayed two peaks of activity 06:00-08:00 and 16:00-19:30 and almost no activity occurred from 20:00 to dawn the following day (Gattermann et al., 2008). This was also found in mice, with those in the laboratory being 'strictly nocturnal' and those in the field being 'partially or completely diurnal' (Daan et al., 2011).

Although such drastic discrepancies have not been seen in *Drosophila*, there are still noticeable variations in behaviour collected under standard laboratory conditions compared to the field. As previously mentioned, variations in behaviour are apparent when more realistic lighting and temperature cycles are used within the laboratory and so is unsurprising there are differences between a wild and artificial settings. Vanin et al. (2012) compared locomotion behaviour of *D. melanogaster* under typical laboratory conditions with behaviour collected under natural conditions in a field setting. In these experiments they collected locomotion under field conditions from April to November 2007-2009, in Leicester, UK. They found several variations in behaviour between these settings including the loss of 'anticipation' preceding lighting events, the replacement of the midday siesta with an increase in activity and the loss of the general crepuscular behaviour of flies typically found within the laboratory. These differences in behaviour were confirmed by De et al. (2013) who also identified the afternoon activity peak. Vanin et al. (2012), were able to reproduce these 'wild type' behavioural traits when simulated natural conditions were recreated within the laboratory. By introducing fluctuating light intensity cycles to recreate more realistic dawn and dusk periods, alongside fluctuating temperature cycles, they were able to mimic locomotion activity within an artificial environment representative of that observed under natural conditions.

In field behavioural rhythms of *D. suzukii*

As researchers become more aware of the behavioural differences between laboratory and natural conditions, some groups have begun to observe behavioural rhythms in field conditions. Evans et al. (2017), observed oviposition behavioural rhythms of laboratory cultured *D. suzukii* while held in semi-field conditions in Georgia, USA. Cultured flies were either deployed in small cages housing loose blueberry or in cages around blueberries still attached to the bush at various locations. The aim of this assay was to compare day versus night and the effect location of the berries (i.e. in the shade lower in the canopy) on oviposition rates. They found that fewer eggs were laid per female, a lower percentage of pupa developed and fewer progeny per female occurred from cages deployed during the day than at night. However, the temperatures experienced during this field trial averaged at 31-32 °C during the day, and the low amount of oviposition was due to no flies surviving from cages deployed in the day. Behavioural patterns of wild populations in field conditions would be the optimum parameters for understanding *D. suzukii* rhythms. However, at the time of writing, only two papers have evaluated the behavioural patterns of wild, unrestricted *D. suzukii* in the field.

Swoboda Bhattarai and Burrack (2015) monitored diurnal attraction to baited traps that were deployed within blackberry crops in North Carolina. Traps were checked hourly and peaks in trap catch occurred from sunrise to mid-morning and from 18:00 to sunset between June and August 2014 and 2015. They also recorded oviposition of wild *D. suzukii* by exposing ripe fruit in either 4 or 8 hour periods for day or night sampling respectively from the 3-4 August 2014. Infestation was low overall but higher emergence occurred from fruit exposed from 18:00-22:00 and 06:00-10:00. While the information provided by this assay is informative, further knowledge regarding environmental conditions, such as temperature, is required to correctly interpret the results. Van Timmeren (2017) recorded the activity of *D. suzukii* in a blueberry crop during the day via direct observations of the pest on the crop. Visual surveys were conducted during 4, two hour periods for 15 minutes between 06:00 and 22:00. During these times average temperature and humidity were also recorded. Significantly more flies were in the crop during the cooler periods of the day in 2015; 20°C between 06:00-08:00 and 24°C between 18:00-20:00. Between 12:00-14:00 and 15:00-17:00 temperatures averaged 30°C. This is the upper limit for *D. suzukii* oviposition as identified by Tochen et al. (2014) who found no oviposition occurred at this temperature. Zerulla at al. (2017) also identified that oviposition activity decreases in temperatures above 28°C and, when provided with a gradient of temperatures, *D. suzukii* showed a preference for egg laying at ~22°C.

As we know, from the examples given above, behavioural rhythms of *D. suzukii* are under the control of the circadian clock. However, how dynamic environmental factors influence and affect these rhythms is not yet known. In order for results in behaviour to be comparable, a greater understanding of the impact of conditions or a standardised methodology is needed to reach clear conclusions.

Oviposition disruption

As egg laying female *D. suzukii* are responsible for spoiling fruit, deterrents to discourage oviposition have been investigated as control options (Erland et al., 2015). Pheromones are complex volatile signals that play a vital role in insect communication through the emitting and detection of chemicals (Sengupta and Smith, 2014, Kohl et al., 2015). In some insects, aggregation pheromones are used to communicate to conspecifics where beneficial communal egg laying sites are situated which improves offspring survival (McCall, 1995). Exploiting attraction to pheromones as a control measure is used for many pest insect species which generally use aggregation or sex pheromones to lure individuals into trapping agents (Yew and Chung, 2015, Sonenshine, 2017). In *Drosophila*, pheromones are also used to communicate in courtship, aggression and aggregation (Wertheim, 2001, Symonds

and Wertheim, 2005, Dwecka et al., 2015, Wicker-Thomas and Hamann, 2008, Enjin and Suh, 2013).

Cis-vaccenyl acetate (*c*VA) is a complex, multifunctional, male specific pheromone used in volatile signalling in most *Drosophila* species (Datta et al., 2008). However, *c*VA appears to have a repellent effect on *D. suzukii*, and when applied to males, resulted in mating disruption. Dekker et al. (2015) found that *D. suzukii* do not produce *c*VA and the ejaculatory bulb which emits the pheromone is atrophied. The receptors that detect *c*VA are functional in *D. suzukii* but they are significantly smaller in size than those in species that communicate through *c*VA volatiles. The authors originally suggest that the reversal in perception of this pheromone could be associated with the male wing spot. *D. subpulchrella,* and *D. biarmipes* are also identified by single wing spots and are found in the *suzukii* group. Dekker et al. (2015) found that *D. suzukii* and *D. subpulchrella*, both of which utilise under-ripe fruit for oviposition, do not produce *c*VA, whereas *D. biarmipes* which oviposits into decomposing fruit does produce *c*VA. They concluded that the loss of cVA function is associated with the preference of under-ripe fruit for oviposition. However, in assays performed by Bernardi et al. (2016) when given a choice of varying fruit ripeness, significantly more *D. suzukii* eggs were laid and adults emerged from ripe and over-ripe fruit compared to ripening. There was also no significant attraction preference to under-ripe fruit in comparison to ripe or over-ripe in approach assays (Keesey et al., 2015). When *D. melanogaster* females lay eggs, they mark optimum areas with aggregation pheromones including *c*VA and cuticular hydrocarbons within redundant male sperm (Dumenil et al., 2016). Therefore it could be that decomposing fruit, which is already inoculated with the eggs of other Drosophila species, deter female *D. suzukii* from ovipositing in due to the presence of *c*VA.

Chemical control and the circadian clock

Currently the primary method in reducing *D. suzukii* populations is chemical control, which has resulted in an increase or “reestablishment” of routine spray programs in fruit crops which previously had established reduced insecticide use through IPM(Roubos et al., 2014). This has disrupted long established IPM programs that combine biological, chemical and cultural control to reduce pest and pathogen pressures (Fountain and Medd, 2015). Also, due to the increasing restrictions on chemical usage and the loss of many key active ingredients though the pesticide approval process, growers will have to rely on other

methods to control this pest (Hillocks, 2012) or ensure chemicals being applied have the highest impact.

It had been widely assumed that applications of chemical control products at peak *D. suzukii* activity time would increase the possibility of contact and result in higher mortality (Shipp and Otton, 1976). Previous work investigating detoxification in *D. melanogaster* had concluded that genes associated with the process were circadian regulated along with periods of susceptibility to specific chemical formulations (Hooven et al., 2009). As detoxification is related to the metabolism of an organism, which is circadian regulated, it is clear why the assumption that detoxification is also circadian regulated arose (Wijnen and Young, 2006).

A paper by Hamby et al, (2013) looked at the susceptibility of female *D. suzukii* to two commonly used pesticides in the USA: malathion, an organophosphate, and danitol (2.4 EC), a pyrethrum; both of which affect the nerve function of insects. Different doses of each pesticide were applied at 4 time points within a day and they found no variation in percentage mortality to pyrethrum between time points. For malathion, the highest tolerance occurred at ZT20 (02:00) defined as the lowest mortality. The lowest tolerance occurred at ZT0 (06:00) which is when the highest mortality occurred. This period of susceptibility occurred during a period of 'higher than average' activity but the authors do state that high activity levels in the laboratory may not always indicate periods of higher plant protection product susceptibility. It appears from this study that activity level may be a contributing factor when combined with the cycling of genes associated with detoxification. As different plant protection products have different modes of action (MOA) they work in a variety of ways (IRAC, 2016). It is, therefore, possible that different MOA's could also vary in susceptibility in relation to how they contact the insect i.e. if it is required to be ingested for it to be affective.

Summary

D. suzukii is one of the largest economic pests the horticultural industry is currently facing. Due to its unique and indiscriminative oviposition behaviour, it has been causing yield loss and higher input costs for soft- and stone-fruit growers, on a global scale. Although there are IPM control methods available to target this pest, their efficacy could be improved by increasing a basic understanding of *D. suzukii* behaviours.

The circadian clock is an organisms' internal pacemaker, which controls behavioural and physiological patterns in processes such as locomotion, oviposition and toxin sensitivity. Various biotic and abiotic cues regulate the clock including light, temperature and also social interaction. The very purpose of the clock is to ensure an organism is in synchrony with its environment, which in turn synchronises individuals to conspecifics. This provides an opportunity to exploit behavioural patterns in a species. In order to do this, we need an understanding of how both biotic and abiotic cues are entraining the circadian clock in *D. suzukii* and therefore influencing behavioural outputs.

From previous literature, it is clear that behavioural patterns within a laboratory setting can vary greatly as the result of inconsistent methodology. While the standard conditions have enlightened us on the function of the circadian clock, they provide very little insight to behaviours under natural or realistic environmental conditions. Ideally behaviour assays would be performed within a natural setting. However, there are many restrictions of working in the field that often prevent behavioural assays being performed under natural conditions. The capability to power automatic recording devices, protect them from inclement weather or even protect experiments from inquisitive wildlife, all restrict the ability to monitor natural behaviour outdoors.

There are examples of discrepancies in behaviours between those observed within a laboratory or in a field setting. However, from the initial results of pervious researchers, these discrepancies can be minimalised when more realistic parameters are used. By introducing fluctuating temperatures and gradual lighting changes, groups have been able to produce 'wild type' behaviours in a laboratory setting. With a greater understanding of how parameters influence behaviours we will be able to identify peaks in key behaviours in relation to environmental conditions.

Once we have addressed how these factors regulate behavioural patterns in *D. suzukii* we can then explore how these behaviours can be disrupted or exploited to provide additional control against this pest. The investigation of how the circadian clock can be exploited to improve plant protection products susceptibility has already begun in *D. suzukii.* However,

the products investigated are not approved for use and so the current results are irrelevant for UK growers.

Having an understanding of the biotic and abiotic factors that can disrupt these behaviours could illuminate additional opportunities to exploit natural behavioural responses. It is intended that we may be able to exploit the behaviour and physiology of *D. suzukii* to enable more effective control with precision monitoring devices and chemical and biochemical controls.

Materials and methods

Over all methods

Drosophila suzukii culture maintenance

D. suzukii cultures were established from a wild Italian strain collected from the Trento area in autumn 2012 and were used through-out this project. Populations of *D. suzukii* were housed at 23°C in a 12:12 L:D cycle within environmental controlled chambers. Lights on at 08:00 and off at 20:00. Cultures were maintained on standard BDSC cornmeal food (100 % dH_2O, 1 % Fisher agar, 9 % table sugar, 9 % precooked ground maize, 2 % baker's yeast, 1 % soya flour, 5 % light spray malt, 0.3 % propionic acid, 0.3 % methyl benzoate dissolved in 3 % 70% ethanol (https://bdsc.indiana.edu) and were transferred into new vials every week. Cultures were mixed every few weeks to prevent genetic bottle necks occurring.

Experiment 1: Investigating the locomotion of D. suzukii under different social conditions and temperature and light cycles

Within this section we identify the determinants of daily rhythms of *D. suzukii* locomotor activity. The impact of gender, space, social housing, temperature, light and the circadian clock on *D. suzukii* locomotor rhythms were investigated. Assays were performed under artificial laboratory conditions or natural, field conditions to identify how the various factors impact behavioural patterns.

Locomotion profiles

Locomotion profiles for *D. suzukii* were created using a Trikinetics *Drosophila* Activity Monitor (DAM) device. This electrical device records activity by movement breaking an infra-red beam. Individual male and female activity profiles were created using a 32 channel individual DAM (Figure 2) system which held glass cuvettes 7cm long with 5mm diameter. Groups of males, females and mixed sex groups were monitored within a DAM population monitor (Figure 3) which held glass vials 15cm long with 30mm diameter. Groups of flies consisted of either single sex groups of 10 males or 10 females, or mix sex groups of 10 males and 10 females. To determine whether space was a factor in the locomotion rhythms, individual males and females were monitored within larger population monitors in some of the conditions. Both individual cuvettes and population vials contained a basic sugar and agar food to sustain the flies during the assessment process.

The DAM devices recorded from either within an incubator or in semi-field conditions. Locomotion was recorded in semi-field conditions from within a well-ventilated Perspex

structure, to protect the equipment from rain, at three times of the year (Table 1). In the laboratory simple (either light or temperature cycles), combined cycles (light and temperature cycles) and semi-field mimic cycles (as identified in the semi-filed locomotion collections) were set within a Percival DR-36VL environmental incubator (Table 2). Photoperiod was taken from recording sunrise and sunset times.

Figure 2. Standard 32 channel Trikenetics individual *Drosophila* Activity Monitor. Glass cuvettes 7cm long with 5mm diameter.

Figure 3. Large 32 channel Trikenetics population *Drosophila* Activity Monitor. Glass vials 15cm long with 30mm diameter.

Table 1. Environmental conditions collected alongside semi-field locomotor assays.

Location	Category	Name	Identification Code	Temperature	Photoperiod
Field	Semi-field	April	Apr SF	Average temperatures 8-15^{o}C	Day length: 12.5:11.5 Civil sunrise: 06:15 Civil sunset: 20:00
Field	Semi-field	June	Jun SF	Average temperatures 13-19^{o}C	Day length: 16.5:7.5 Civil sunrise: 04:00 Civil sunset: 22:00
Field	Semi-field	August	Aug SF	Average temperatures 14-23^{o}C	Day length: 15.35:8.65 Civil sunrise: 04:45 Civil sunset: 21:20
Field	Semi-field	October	Oct SF	Average temperatures 8-14^{o}C	Day length: 11:13 Civil sunrise: 06:45 Civil sunset: 18:45

Table 2. Environmental conditions used in Percival DR-36VL environmental chambers.

Category	Name	Identification Code	Temperature	Photoperiod
Simple	DD 23°C	DD23	Constant 23°C	Constant darkness
Simple	12:12 23°C	12:12 23	Constant 23°C	12:12 light: dark
Simple	DD 11-22°C	DD 11-22	Ramping 11-22°C Min: 04:00. Max: 12:00	Constant darkness
Simple	16:8 23 °C	16:8 23	Constant 23°C	16:8 light: dark. On 04:00 Off 20:00
Combined cycles	Long day	SLD	Ramping 11-22°C. Min: 04:00. Max: 12:00	17.5:6.5 Stepping lights. Half-light: 04:30 Full light: 05:00 Half-light: 21:30 Darkness: 22:00
Combined cycles	Short day	SSD	Ramping 11-22°C. Min: 04:00. Max: 12:00	10.5:14.5 Stepping lights. Half-light: 06:30 Full light: 07:00 Half-light: 16:30 Darkness: 17:00
Semi-field mimic	April mimic	Apr M	Ramping 8-15°C Min: 05:30 Max: 14:00	13.5:10.5 Stepping lights. Half-light: 05:30 Full light: 06:00 Half-light: 18:30 Darkness: 17:00
Semi-field mimic	June mimic	Jun M	Ramping 13-19°C Min: 04:00 Max: 14:00	17.5:6.5 Stepping lights. Half-light: 04:30 Full light: 05:00 Half-light: 21:30 Darkness: 22:00
Semi-field mimic	August mimic	Aug M	Ramping 14-23°C Min: 05:00 Max: 14:00	16:8 Stepping lights. Half-light: 05:00 Full light: 05:30 Half-light: 20:30 Darkness: 21:00
Semi-field	October	Oct M	Ramping 8-14°C	12.5:11.5 Stepping

mimic	mimic	Min: 06:45 Max: 14:00	lights. Half-light: 06:45 Full light: 07:15 Half-light: 18:45 Darkness: 19:15

Analysis

For each recording across all combinations of sex, group and condition, data was collected for a minimum of 6 days. The ClockLab Circadian Analysis software for circadian biology (ACTi Metrics) was used to analyse the data and produce activity profiles (the average activity of the assessment period in 24 hours) for each set of data. In the laboratory a minimum of 50 individual males or females that survived the whole assessment period were batch analysed to create one average profile per sex. A minimum of 5 groups were used. In the semi-field collections the total number that survived the assessment period was used for the analysis which resulted in a variation in sample size.

Average activity counts per hour per fly were calculated by dividing hourly activity by the number of flies in the sample group to enable comparison between all social groupings and environments. A non-parametric, pairwise comparison of average activity counts per hour per fly was performed in SPSS.

To determine the impact of social housing on time keeping ability in constant conditions (DD 23ºC), relative rhythmic power (RRP), was calculated from chi-square periodogram analyses in CLOCKLAB by dividing the ‘power’ by the ‘significance’ threshold values for each rhythmic fly (using a threshold of p=0.01) manually in Excel. In the virtual groups, RRP was manually calculated from the chi-squared periodogram. In cases where the program generated an RRP less than 1, values were replaced with 1’s as this is the lower limit of minimum value generated by the ClockLab software. RRP values between 1 and 1.5 were considered to represent weak rhythmicity. The period length (i.e. the duration of locomotor behavioural cycles) was identified by the ClockLab software. A one-way ANOVA and a Turkey’s multiple comparison was performed in GraphPad Prisim 7.03 on both these parameters.

Experiment 2: Can we predict oviposition rhythms in the field in laboratory based experiments?

Within this section we aimed to investigate rhythms in oviposition behaviour of wild *D. suzukii* under natural conditions in the field. Environmental parameters were also recorded to decipher how they influence these rhythms. Assays were then performed on laboratory cultures, housed under artificial conditions mimicking the temperature and light cycles, to see if these patterns were reproducible and rhythmic.

Field oviposition

Field oviposition rhythms were collected at times in the year to coincide with specific crop or *D. suzukii* population events. Oviposition assays were performed in an unsprayed, protected, strategic cherry orchard (Sweetheart and Penny cv.) located at NIAB-EMR, Kent, UK: cherry at ripening (June, start date 06/06/18 and August, start date 02/08/16) and end of cropping season (October, start date 02/10/16). *D. suzukii* were collected from monitoring traps (Droso trap, Biobest) in the vicinity of the oviposition assay sites baited with the Char-Landolt four component lure (Cha et al., 2014). Females were dissected to determine fecundity at the beginning of the experiment by identification of mature ovaries and eggs (Gerdeman and Tanigoshi, 2012). Green delta traps were modified by covering the open sides with a 3 mm wire mesh to prevent entry of larger animals that may utilise the fruit (Figure 4 and Figure 5). Traps were hung on cherry trees 1.5 m above the ground in shaded areas and spaced 15 m apart. Single ripe cherries, donated by G. H. Dean and Norton Folgate (August), or raspberries donated by Driscoll's (October) of randomised varieties were placed within the trap. Fruit type was depended on seasonal availability. After two hours the fruit was removed and transferred to a ventilated 70 ml specimen container (7 cm high x 5cm diameter, polypropylene). Fruits were replaced within the trap every two hours throughout the day from roughly 15 minutes before civil sunrise to 15 minutes before civil sunset (*https://www.timeanddate.com/sun/uk/maidstone*) for three days. Fruit was offered throughout the night but was not changed. Data loggers (Lascar Electronics Ltd.) recorded temperature and humidity throughout. The samples were stored for 21 days at 25^{o}C under a 16:8 light: dark cycle within a quarantine facility at NIAB-EMR. Samples were frozen and flies that emerged from the fruit were identified as either male *D. suzukii*, female *D. suzukii* or other and were counted. Did the flies emerge after freezing?

Figure 4. Modified green delta trap with new side entry point. Petri dish containing a single cherry is the egg laying site for the experiment

Figure 5. Modified green delta trap from side entrance covered with a 5mm wire mesh to prevent entry of larger insects and birds.

Laboratory assays

General methods

As temperature and light cycles varied with experiment on commencing the assays, light and temperature cycles were modified within the chambers accordingly and are discussed in each section. For those conditions with a light phase over 12 hours a minimum of two chambers were used and were staggered to different 'time zones' enabling samples to be collected in a 12-hour time span and increasing replication. *D. suzukii* were acclimatised to experimental conditions for a minimum of 72 hours before starting assessments. Twenty-four hours before the start of laboratory trials, flies were removed from the chambers while in the photo phase. Three to seven-day old flies were immobilised on a CO_2 pad (Flystuff.com) for a maximum of two minutes while sexed and transferred by the wing with soft forceps to experimental arenas, which varied with assay. In all assays' it was presumed that flies had mated prior to the start of the experiment. Flies were a minimum of 4 days old as Revadi et al., (2015) found that reproductive mating occurred in flies 2.5 days after emergence.

Relationship between oviposition and adult emergence/ 12:12 LD 23°C

Groups of males and females were transferred to 70 ml specimen containers, as used in field oviposition assays, containing 10 ml of BDSC cornmeal media. Flies were subjected to constant 23°C under a 12:12 LD cycle. At lights on and lights off, flies were transferred to a new specimen container of media by tapping from the old container to the new. Eggs were counted under a microscope (x6 magnification) and the specimen container returned to the environment chamber. The same flies were transferred 6 times resulting in 3 days of assessments with 3 repetitions of light and dark counts. After 21 days the emerged adults were counted and egg to emergence survival assessed. The results from this assay were also used to deduce the light vs dark egg laying behaviour at a constant temperature (identified as 12:12 LD, 23°C in the results and discussion).

Basic environmental cycle/17:7 LD 17/11°C

Groups of male and female *D. suzukii* were transferred to insect cages (17.5 x 17.5 x 17.5 cm, BugDorm-41515) housed within the environment chambers. Flies were subjected to a 17:7 LD cycle. Chambers were programmed with a day temperature of 17°C and a night temperature of 11°C with temperatures changing at lighting events. All cages were provisioned with water *ad libitum*, via a large Petri dish containing a soaked cotton wool reservoir, and food, a Petri dish (55 mm) of the BDSC cornmeal media. On commencing the trial, the Petri dish of media was replaced hourly for three days. Each dish was inspected under a microscope (x6 magnification) and the number of eggs counted.

Recreated field conditions in the laboratory

Environment chambers were programmed with temperature cycles and lights-on and off times according to average field conditions during the reproductive oviposition field trials (June 11-22 ºC, 18:6 L:D cycle 04:30 half-light, 05:00 full light, 21:30 half-light, 22:00 full-dark; August 14-32ºC, 16:8 L:D cycle, 05:00 half-light, 05:30 full-light, 20:30 half-light, 21:00 full-dark; October 9-18 ºC, 12.5:11.5 L:D cycle, 06:45 half-light, 07:15 full-light, 18:45 half-light, 19:15 full-dark). The maximum temperature used in the August conditions was reduced by 2ºC due to avoid high heat-associated mortality observed in preliminary trials. At lights-on and off, half of the lighting banks in the chamber were changed 0.5 h prior to the other half to create a stepped transition. Groups of male and female *D. suzukii* were transferred to insect cages, as above, and housed within the environment chambers. All cages were provisioned with water *ad libitum* and a Petri dish (55 mm) of the BDSC media. On commencing the trial, the Petri dish of media was replaced every two hours for three days. Egg laying was assessed throughout the light phase only. Media was offered through the dark phase but was not changed or assessed. Each dish was inspected under a microscope (x6 magnification) and the number of eggs counted.

Relationship between locomotion and oviposition in the laboratory

Groups of 10 male and 10 female *D. suzukii* were loaded into LAM25 monitors (as discussed in experiment 1) in 25 mm x 95 mm glass vials containing 10 ml of a set sugar and agar food (dH_2O, 5% sugar and 1% agar) sealed with a breathable cotton bung. Flies were taken from mixed sex cultures and were between 3- 7 days old at the start of the assay and presumed to have mated. Flies were immobilised and held on a CO_2 pad before being transferred by the wing with soft forceps to the mouth of the vial. LAM25 monitors were housed in the Percival DR-36VL environment chambers under the conditions used in the three recreated field oviposition laboratory trial. Locomotion was monitored for a minimum of 6 days after a 24 hour 'settling' period.

Analysis

A linear regression analysis was performed to establish correlation between the number of eggs laid and subsequent adult emergence under laboratory conditions. Hourly oviposition in the laboratory and reproductive oviposition in the field was calculated to make results comparable across experiments. In those experiments when dark phases were assessed (12:12 LD 23°C, 17:7 LD 17/11°C in the laboratory and both field assays) Mann Whitney U test were performed for light vs. dark counts within each assay. Light counts/h were divided by dark counts/h to calculate the fold increase of light to dark oviposition rates. For both the

August and October field and laboratory assays, normalised counts were calculated as a fraction of the total counts for each individual experimental day to allow for variation in population densities. A Mann Whitney U test was performed for pairwise comparisons of laboratory vs. field for normalised oviposition dependent on both time and temperature. Temperatures were divided into ranges and normalised oviposition analysed with a Kruskal-Wallis test to identify optimal temperature conditions. Statistical analysis was performed in SPSS.

For locomotion analysis, only groups in which all flies survived the full assessment period were used, resulting in a minimum of 6 groups per condition being analysed. Locomotion rates were analysed with ClockLab software through Matlab. Batch analysis was used to generate average locomotion activity from the sample groups. Average counts per minute and SEM were generated in ClockLab in 30-minute bins and combined into 2-hour intervals to match the format of the oviposition data.

Experiment 3: How will the presence of another species affect oviposition behaviour in D. suzukii?

In the work reported here the inter-species competition between *Drosophila* suzukii and *Drosophila* melanogaster adults was investigated. The impact of *D. melanogaster* had on both the emergence of *D. suzukii* offspring and the number of eggs laid by *D. suzukii* females was observed to distinguish whether the presence of another species is detrimental to *D. suzukii* population growth.

Next generation emergence

A competition experiment was conducted in a contained chamber within the insect quarantine facility at NIAB-EMR in the 25°C in a 16h: 8h light/dark cycle. A Petri dish of either cornmeal (x1 experiment) media or fresh raspberries (x2 experiments) was transferred into a 12 x 7 x 7 cm ventilated Perspex box. *D. suzukii* and *D. melanogaster* were held on a cold table to immobilise them for a maximum of three minutes while sex was identified. For both species, males were identified by the presence of sex combs and females by the oviscapt morphology. The following methodology is visualised in Figure 6. For the first inoculation, five female and two male adults (3-7 days old) were transferred to the Perspex boxes. In treatment 1, *D. melanogaster* adults were added and for treatments 2 and 3, *D. suzukii* adults were added. For each experiment, six replicates of each of the three treatments were done. Immobilised flies were held by the wings and transferred individually with soft forceps to the base of the ventilated box. Flies were not placed on the substrate as in some cases females expel eggs when chilled and this would have interfered with the objective of the experiment. Lids were returned to the Perspex box and sealed closed with electrical tape. All boxes were checked 10 minutes after flies had been added to ensure all were mobile and had recovered from chilling.

Forty eight hours after the first inoculation, all adult flies were removed from the boxes with an electric pooter. A new, blank Petri dish of either the cornmeal media or raspberry fruit was added to the box. For all treatments, boxes now contained two substrate dishes: one pre-inoculated and one with blank media. Flies were once again immobilised on a cold table. For the second inoculation, five female and two male *D. melanogaster* adults were added to treatment 2, five female and two male *D. suzukii* adults were added to treatments 1 and 3. As before, lids were sealed with electrical tape and were checked 10 minutes after flies had been added to ensure recovery.

Forty eight hours after the second inoculation all flies were removed from the boxes with an electric pooter. Single Petri dishes were then moved into individual ventilated Perspex

boxes (6 x 6 x 6 cm) and stored at 25°C for the remainder of the experiment. As *D. melanogaster* and *D. suzukii* have different development times the duration of the experiments was critical. At 25°C *D. melanogaster* takes a minimum of 7 days from egg laying to the next generation adult emergence and a further 48 hours before newly emerged females are able to oviposit viable eggs (Tyler, 2000). This would mean a third generation would emerge after a minimum of 16 days. The minimum development time for *D. suzukii* at 25°C is 11 days (Hamby et al., 2016). To prevent counting the third generation of *D. melanogaster* but to allow enough time for *D. suzukii* to emerge, Petri dishes were frozen 15.5 days after the first inoculation.

After freezing, counts of emerged *D. suzukii* and *D. melanogaster* adult offspring were done under a dissecting microscope to identify species and sex.

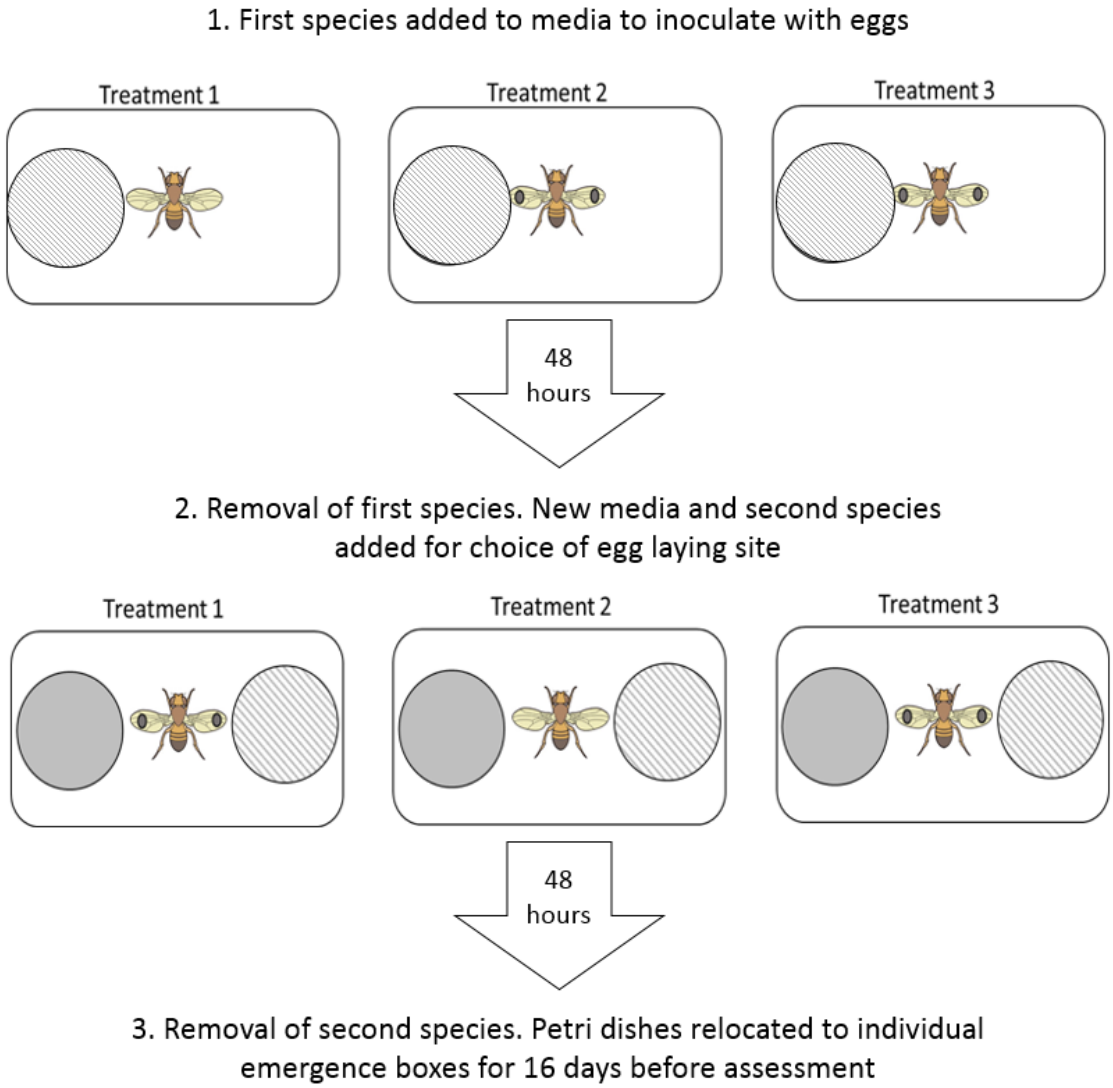


Figure 6. Visualisation of the inoculation process. *Drosophila* with spots on the wing indicate *D. suzukii* inoculation. *Drosophila* with-out spots on the wing indicate *D.*

melanogaster. **Dark grey disk represents substrate with eggs from first species inoculation. Light grey disks represents new, blank, substrate containing no eggs initially**

Female oviposition choice

To identify whether any reduction in *D. suzukii* emergence was due to the number of eggs laid initially, an oviposition choice assay was set up. Twenty Petri dishes containing cornmeal medium were transferred into a 20 x 20 x 20 cm bug dorm. Fifty mated male and female *D. melanogaster* were added to the bug dorm. Forty eight hours after the inoculation, all Petri dishes were removed from the bug dorm and the number of egg cases (hatched and unhatched) counted under a microscope at ×12 magnification. Ten Petri dishes containing between 6 and 20 egg cases were transferred individually into 12 x 7 x 7 cm ventilated Perspex boxes. This was to ensure that the medium was not too densely populated initially. A new, blank Petri dish of the cornmeal medium was added to each box. Boxes now contained two Petri dishes: one pre-inoculated and one with blank medium. *Drosophila suzukii* individuals were immobilised on a cold table before five female and two male adults (3–7 days old) were transferred, by the wing, to each of the Perspex boxes. Lids were sealed with electrical tape and were checked 10min after flies had been added to ensure recovery.

Forty eight hours after the *D. suzukii* inoculation, flies were removed from the boxes and the total number of egg cases counted. The number of egg cases counted after the removal of *D. melanogaster* was subtracted from the total number of egg cases after the removal of *D. suzukii* to obtain the number laid by *D. suzukii* in the pre-inoculated Petri dishes. In the blank Petri dishes, egg cases were presumed to be *D. suzukii* as they are immobile and had not been exposed to any other egg-laying females. Larval counts were not taken as larvae are mobile and could have migrated from one dish to the other.

Analysis

Counts for each species, experiment and treatment (inoculated or blank substrate) were analysed separately using analysis of variance (ANOVA) to compare relative numbers of D. suzukii and D. melanogaster in each substrate for each treatment. A square-root transformation was used to stabilise for variance. Strictly the experiments were laid out as split-plot designs with pre/post counts. However, when they were analysed as such, there was no evidence that the variance between dishes was larger than the variance between

the repeat measurements on the same dish, so the “paired” nature of the design was ignored and within- and between-dish variances were pooled. This also gave more degrees of freedom for significance testing.

The difference between the number of D. suzukii eggs in the pre-inoculated and blank media was analysed using a paired t-test.

Experiment 4: Is there any impact of the circadian clock on plant protection product susceptibility?

To determine whether time of application influences mortality, sub-lethal doses of cyantraniliprole, lambda-cyhalothrin, pyrethrum and spinosad were directly applied to groups of adult *D. suzukii* at two time points within 24 hours. We also investigated the impact of sub-lethal and lethal doses on *D. suzukii* mortality, oviposition and offspring survival over time.

Spray arena set up

A 4.5 cm filter paper (Whatman 5) was placed within a 5 cm glass Petri dish. A cigarette filter (Swan, slim filter tip), soaked in a sugar water solution (10 g white sugar in 100 ml distilled water), was placed upon the filter paper. Three to seven-day old *D. suzukii* from mix sex cultures, were anaesthetised on a CO_2 pad (Flystuff). Six males and 6 females were transferred to the Petri dish. The Petri dish (spray arena) was covered with a 4 mm mesh to prevent the flies from escaping. Flies were allowed to recover for a minimum of 10 minutes before spray treatments were applied.

LC50 identification

A Burkard bench top sprayer (Computer Controlled Table top Sprayer, Burkard Scientific Ltd, Uxbridge, UK) was calibrated before each application resulting in varying equivalent litres per hectare. However, the output was within the range of 400-600 L/ha, and within the label requirements of each product (Table 3). The maximum field rate (FR) dose for cherry or strawberry of pyrethrum (Pyrethrum 5C), lambda cyhalothrin (Hallmark), cyantraniliprole (Exirel) or spinosad (Tracer) were prepared. Serial dilutions were made, initially, from 100% to 6% FR but additional dilutions were used in pyrethrum and cyantraniliprole where mortality was high at the low doses. Dilutions were made no more than 30 minutes before direct application by the benchtop sprayer. Sprays were applied to a minimum of 6 groups of *D. suzukii* adults at a time (each group contained 6 males and 6 females). A control of distilled water was applied in addition to each pesticide. Applications of dose were made in ascending order starting with the control. After application, flies were allowed to recover for 10 minutes within the arena. After recovery, flies were immobilised with CO_2 before being transferred to a ventilated 70 ml specimen container (7 cm high x 5 cm diameter, polypropylene Sarstedt) containing standard BDSC media. Flies were then maintained as in 16:8 LD at 23°C

Table 3. Pesticide treatments, active ingredient and dilution range of field rate (FR) used to spray *Drosophila suzukii.*

Active ingredient	Trade name and company	Equivalent of water volume used (L/ha)	Maximum field dose (ml/ha)	Dilution range of FR (%)
Cyantraniliprole	Exirel (Dupoint)	580	1125	0.33-50
Lambda-cyhalothrin	Hallmark Zeon® (Syngenta)	400	75	6-100
Pyrethrum	Pyrethrum 5C (Agropharm Ltd)	530	2400	1.5-25
Spinosad	Tracer® (Dow AgroScience)	580	150	6-100

Assessments of mortality and survival were made 24 hours after spray application and the number of eggs laid were counted. At this point, all live flies were transferred to a new ventilated specimen container, with the BDSC media, and returned to the environment chamber. Dead flies were discarded. The original specimen container was returned to the environment chamber to allow any eggs laid to develop through to adult emergence. These assessments and transfers were made at 24, 48, 72, 96 and 168 hours after spray application. Pupal and offspring emergence counts were taken 14 and 21 days, respectively, after parent flies were removed from the specimen container and survival of each stage assessed.

Chrono-toxicity

Environment chambers were programmed with a fluctuating temperature and light cycle, mimicking early summer environmental conditions that had been recorded within a cherry orchard in South-Eastern England, in 2015 (11-22ºC 17.5:6.5 L:D). Flies were held under these conditions for a minimum of 3 days before sprays were applied. This was done to entrain flies to these conditions and ensure the flies circadian clocks had synchronised to the new environmental cues. Fifteen minutes before peak or trough temperature (22 °C at 12:00 and 11 °C at 04:30, respectively) flies were removed from the environment chambers.

For the trough temperature, at 04:30, flies were removed from the chamber during darkness and covered with a black-out fabric. All natural and artificial light was prevented from illuminating the fume hood and only red light was used while transferring and spraying the flies to prevent affecting the circadian clock. Following the method in spray arena set up, flies were transferred to the spray arenas. At peak and trough temperature the LC50 dose for each pesticide was applied using the Burkard sprayer as identified by the dose response assay. A water control was also applied for comparison. After application, flies recovered for 10 minutes within the arena were then immobilised with CO_2 before being transferred to a ventilated 70 ml specimen container containing standard BDSC media. Flies were then maintained within the environment chambers.

Assessments of mortality and survival were made 24 hours after spray application and the number of eggs laid was counted. Assays were repeated twice and results combined for analyses.

Analysis

For all analyses, each pesticide was compared to the distilled water control. GLM with binomial distribution and a logit link was used to assess the mortality of total flies (male and female combined) for both dose response and chrono-toxicity data. Probit analyses was performed using the PROBIT ANALYSIS procedure with a logit link in Genstat (VSN International 2015. Genstat for Windows 18th Edition. VSN International, Hemel Hempstead, UK. Web page: Genstat.co.uk). Probit analyses were performed to identify the LC50 for each pesticide 24-168 hours after application.

All egg counts, for both dose responses and chrono-toxicity, were analysed using a GLM with a Poisson distribution and a logit link. Where egg counts per female were assessed, the same analyses were performed with the addition of LOG number of females as an offset.

Offspring analyses of pupa per egg, adult per egg and adult per pupa were analysed using a GLM with a binomial distribution and a logit link.

Results and discussion

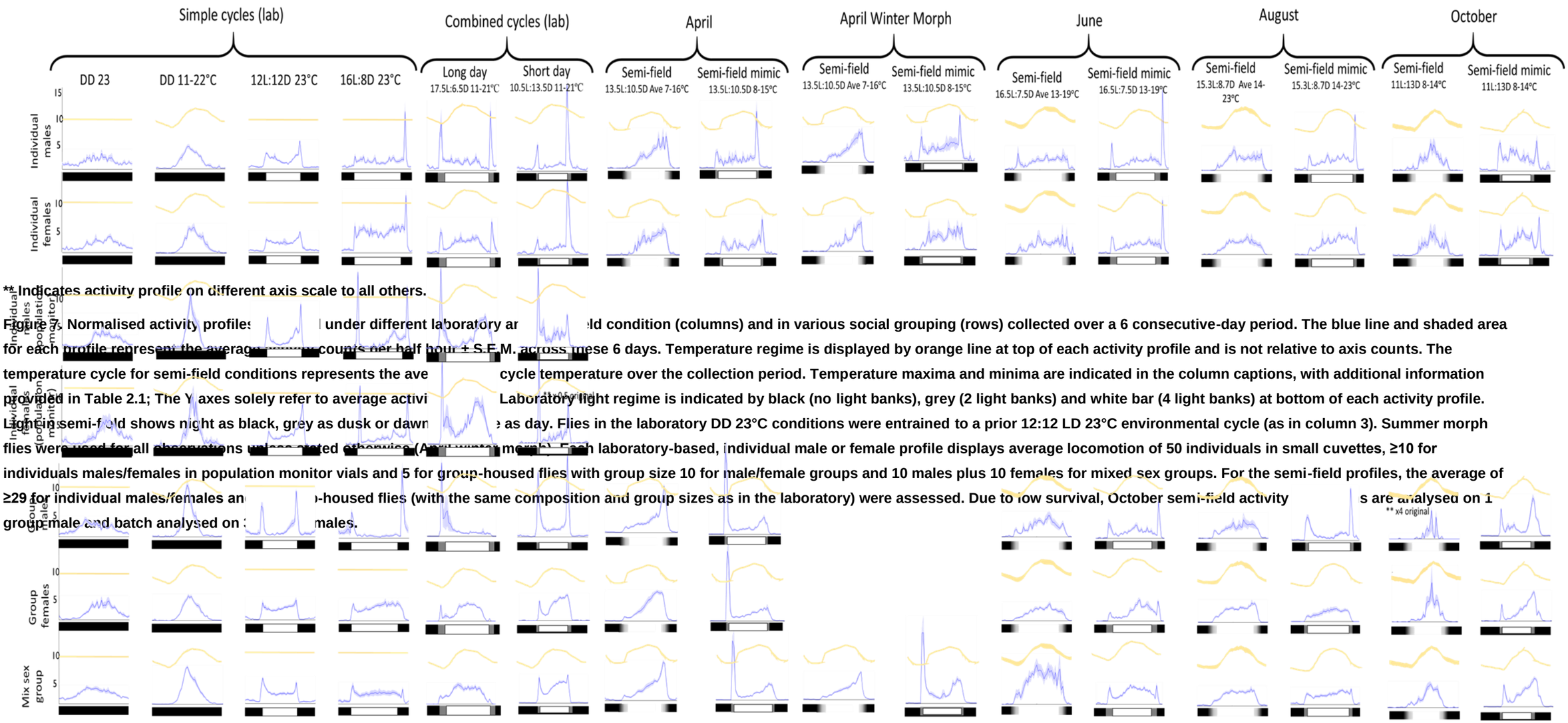
Experiment 1: Investigating the locomotion of D. suzukii under different social conditions and temperature and light cycles

Activity profiles

From the range of social situations evaluated across all environmental conditions, sex composition, space and setting influence locomotion behaviour as seen in locomotion profiles (Figure 7). Activity profiles are visual representations of locomotion activity, monitored over a period of days is then averaged into a 24-hour period. When individual flies were housed in the narrow cuvettes they frequently displayed a different locomotion profile than those in the large population vials. Typically, in the lab-based assays, individual male and female flies as well as group-housed males displayed behavioural profiles with more prominent spikes in activity at lights-on and lights-off than groups of females or mix sex groups under the same environmental conditions. The mix sex groups and female group's activity peaks follow rise and fall corresponding to temperature cycle. Groups of males resemble individual males in general activity pattern.

Environmental conditions had a great impact on *Drosophila* behavioural output with previous groups already highlighting the variation in activity when exposed to 'natural' conditions (Vanin et al., 2012, De et al., 2013, Green et al., 2015). When collected in semi-field conditions and exposed to natural light and temperature cycles all social housings displayed very similar profiles, unlike the laboratory assayed flies which is discussed above. In the laboratory collected profiles (i.e all but the semi-field), when a light cycle was used, most activity occurred during the photo phase and very little occurred during the dark phase. When lighting was removed, activity level followed the temperature cycle if present.

In the semi-field and their corresponding lab mimic collections the female groups and the mix sex groups are most visually similar in activity profile. Meaning that if appropriate environmental conditions are used it is possible to gain an understanding of locomotion behaviours of flies exposed to natural conditions from lab-based assays.



**** Indicates activity profile on different axis scale to all others.**

Figure 7. Normalised activity profiles under different laboratory and field condition (columns) and in various social grouping (rows) collected over a 6 consecutive-day period. The blue line and shaded area for each profile represent the average activity counts per half hour + S.E.M. across these 6 days. Temperature regime is displayed by orange line at top of each activity profile and is not relative to axis counts. The temperature cycle for semi-field conditions represents the average cycle temperature over the collection period. Temperature maxima and minima are indicated in the column captions, with additional information provided in Table 2.1; The Y axes solely refer to average activity counts. Laboratory light regime is indicated by black (no light banks), grey (2 light banks) and white bar (4 light banks) at bottom of each activity profile. Light in semi-field shows night as black, grey as dusk or dawn and white as day. Flies in the laboratory DD 23°C conditions were entrained to a prior 12:12 LD 23°C environmental cycle (as in column 3). Summer morph flies were used for all observations unless stated otherwise (April winter morph). Each laboratory-based, individual male or female profile displays average locomotion of 50 individuals in small cuvettes, ≥10 for individuals males/females in population monitor vials and 5 for group-housed flies with group size 10 for male/female groups and 10 males plus 10 females for mixed sex groups. For the semi-field profiles, the average of ≥29 for individual males/females and 5 for group-housed flies (with the same composition and group sizes as in the laboratory) were assessed. Due to low survival, October semi-field activity profiles are analysed on 1 group male and batch analysed on 3 males.

When both light and temperature cycles were removed for the DD 23ºC conditions, individual males monitored in small cuvette appear to become arrhythmic after 48 hours based on the profile of the actogram (Figure 8a). Individual females in small cuvettes appear to maintain some rhythmicity for 3 days before it is lost. Individual males and females monitored in population vials appeared to remain rhythmic for 5 days. Within the male groups, rhythmicity is maintained for 5 days before the amount of activity through the 'night' increases. In groups of females and mixed sex groups, although there is an increase in night activity, they continue to have peak activity in the 'day time' for the 7-day assessment period. In the relative rhythmic power (RRP) there were significant differences between the social groupings ($F(9,107) = 17.82$, $p < 0.0001$) under the DD 23ºC conditions (Figure 8b). The RRP value is a numerical scale that indicated rhythmic strength; 1-1.5 = weakly rhythmic and >1.5 rhythmic. Virtual group males had the lowest RRP value (1.1). Individual males and females in both the large vials and small cuvettes and virtual female and virtual mix sex groups were classed as weakly rhythmic. The three actual groups were all classed as rhythmic with group females displaying the strongest rhythm (2.1), followed by mix sex groups (1.9) and finally group males (1.6).

The period length was also significantly different between the social groupings in these conditions ($F(9,107) = 3.708$, $p < 0.0004$) (Figure 8c). Virtual group males had the longest period length (30.2 hours). Individual males, virtual group females, individual females and virtual mix sex groups ranged within 27.7 and 24.6 hours. Group males had the shortest length (23.3 hours) followed by group females and individual females in population monitors (both 23.6 hours) and mix sex groups (23.75).

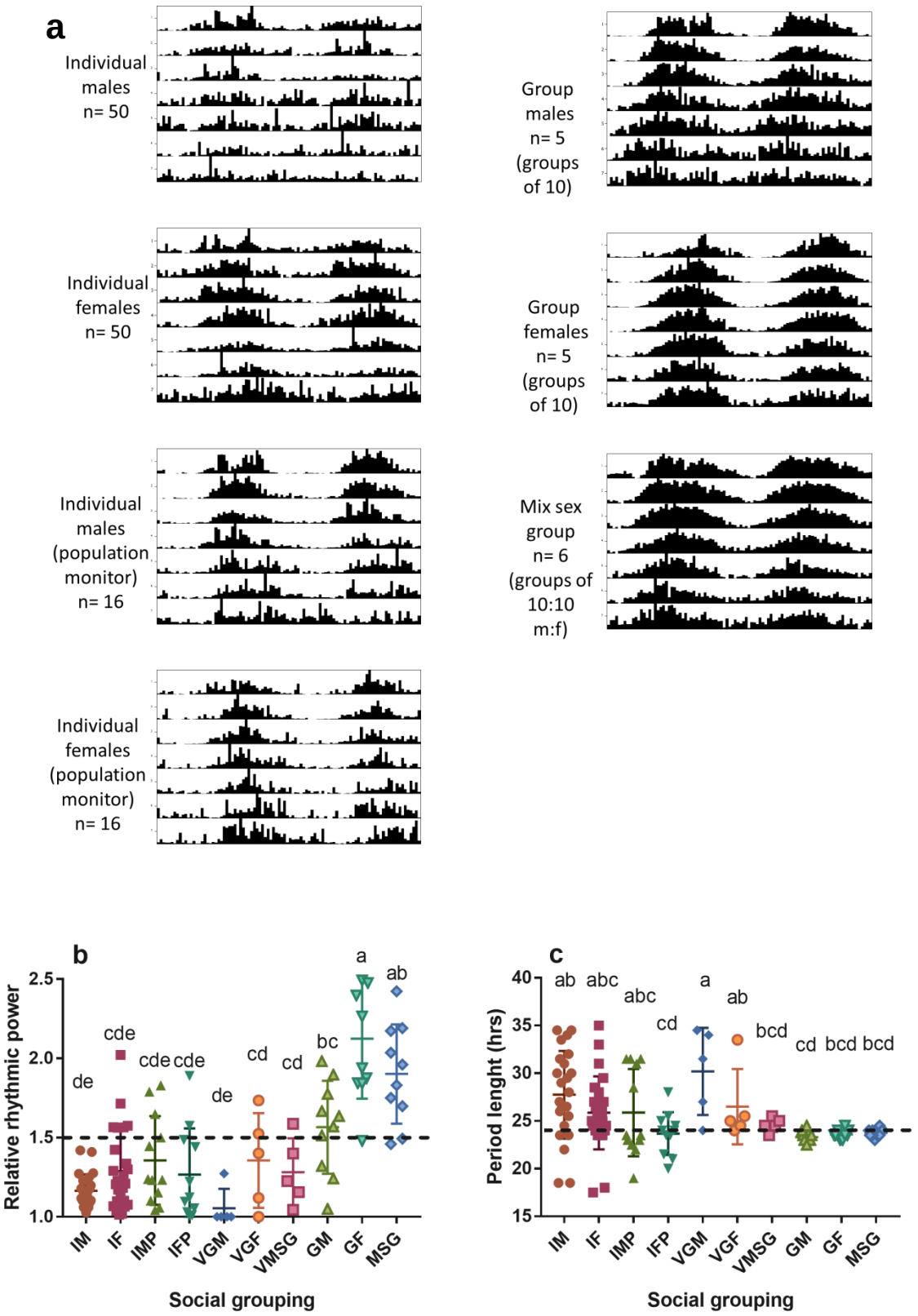


Figure 8 Actogram, RRP and period length of locomotion activity in various social grouping under constant darkness and constant 23ºC. (a) Actogram collected over a 7-day period in 30-minute bins. Each actogram line displays 48 hours in which the last 24 hours is repeated as the beginning of the following row. Single sex groups

display average locomotion of 5 groups of 10 individuals. Individual males and females are the average or 50 individuals. Individuals in population monitors are the average of 16 individuals. Mix sex groups displays average locomotion of 6 groups of 10 males and 10 females in population monitors. (b) Relative rhythmic power (RRP) value indicates strength of rhythmicity. 1-1.5 = weakly rhythmic and >1.5 rhythmic. Black dashed line at 1.5 shows cut off between weakly rhythmic and rhythmic RRP. (c) Period length of the various social groupings in hours

Activity counts per hour

There were significant differences in the average activity counts per hour per fly in relation to environmental condition in all social housing (all $p < 0.000$) (Figure 9). Both individual females in population monitors and group housed females displayed the widest variation in activity counts per hour per fly between environments. Individual males and group housed males typically had the lowest activity counts per hour per fly within each environmental condition Winter morph activity counts, although displayed, were not included in the overall counts per hour per fly statistical analysis as only two groups were observed (Figure 10).

Summer morph flies appear to be more active in the April semi-field conditions than the winter morphs. The winter morphs were more active in the mimic conditions within the laboratory.

Within each environmental condition, there were significant differences in average activity counts per hour per fly between social housing (all $p < 0.008$) excluding one condition (Figure 11). Under DD 23ºC there were no significant differences in counts per hour per fly between the social groupings. Groups of females display the highest activity counts per hour per fly in 6 out of the 16 conditions.

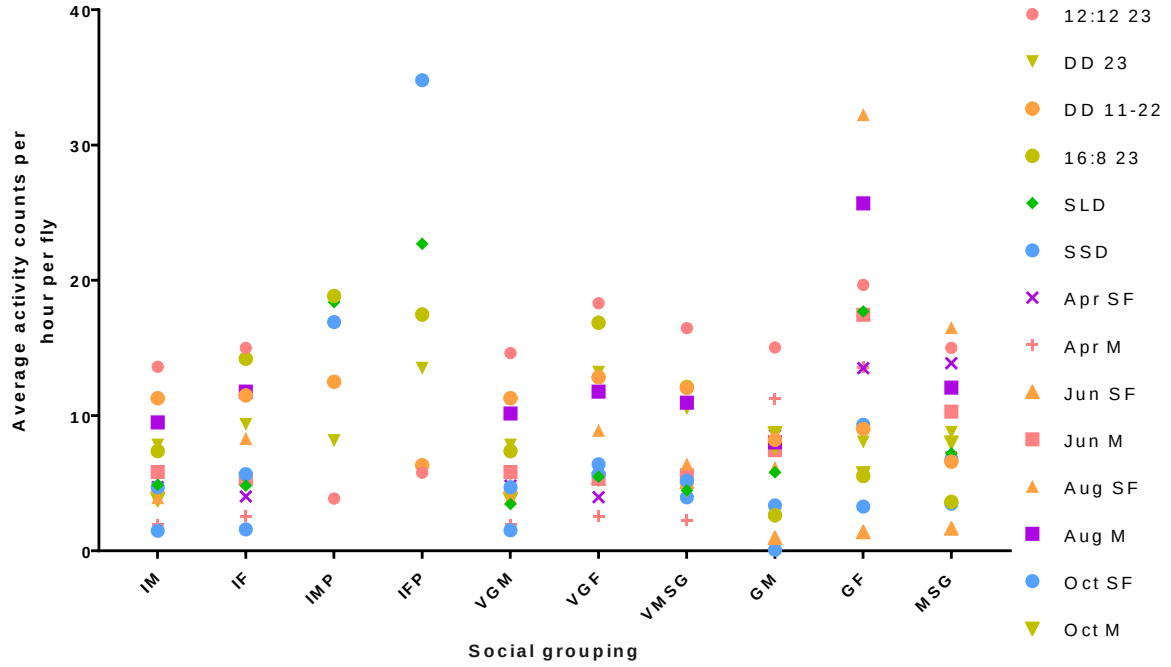


Figure 9. Mean activity per hour per fly of individual males (IM), individual females (IF), individual males in population monitors (IMP), individual females in population monitors (IMF), virtual groups of males (VGM), virtual groups of females (VGF), virtual mixed sex groups (VMSG) groups of males (GM), groups of females (GF), mix sex groups (MSG under various environmental conditions including semi-field (SF), mimic (M) and standard laboratory conditions.

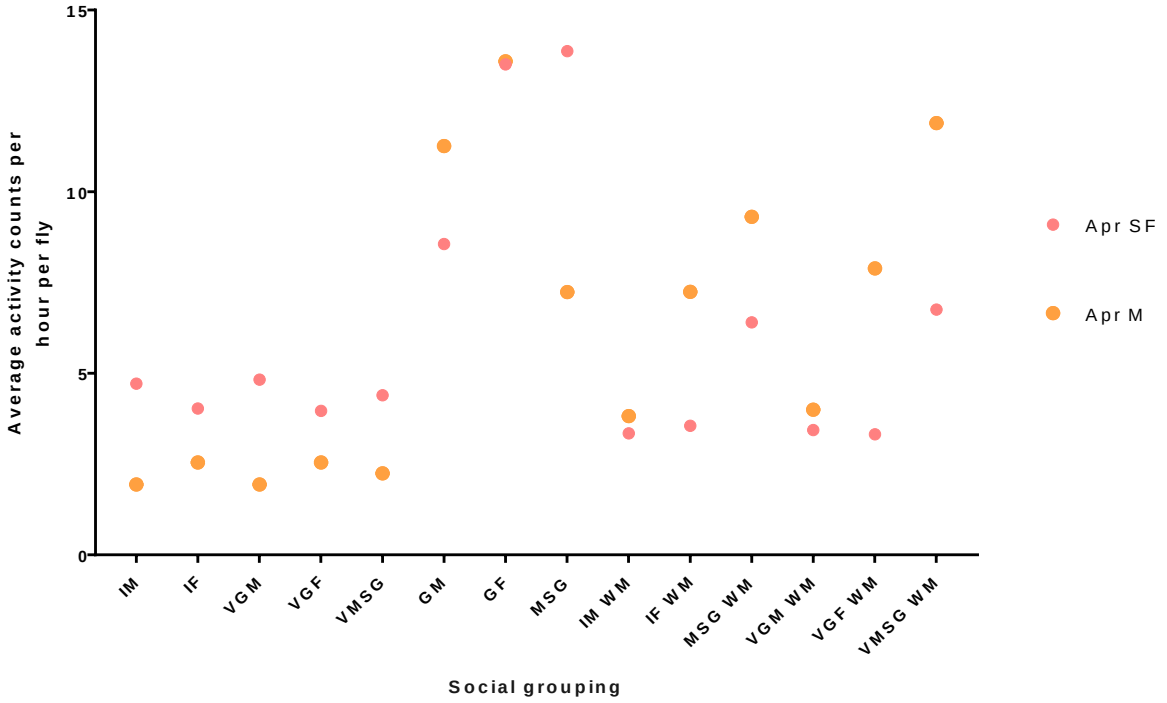


Figure 10. Mean activity per hour per fly of individual males (IM), individual females (IF), individual males in population monitors (IMP), individual females in population monitors (IMF), virtual groups of males (VGM), virtual groups of females (VGF), virtual mixed sex groups (VMSG) groups of males (GM), groups of females (GF), mix sex groups (MSG), individual males winter morph (IM WM), individual females winter morph (IF WM), mix sex groups winter morph (MSG WM), virtual group males winter morph (VGM WM), virtual group females winter morph (VGF WM) and virtual mix sex groups winter morph (VMSG WM) under April semi-field and April mimic environmental conditions.

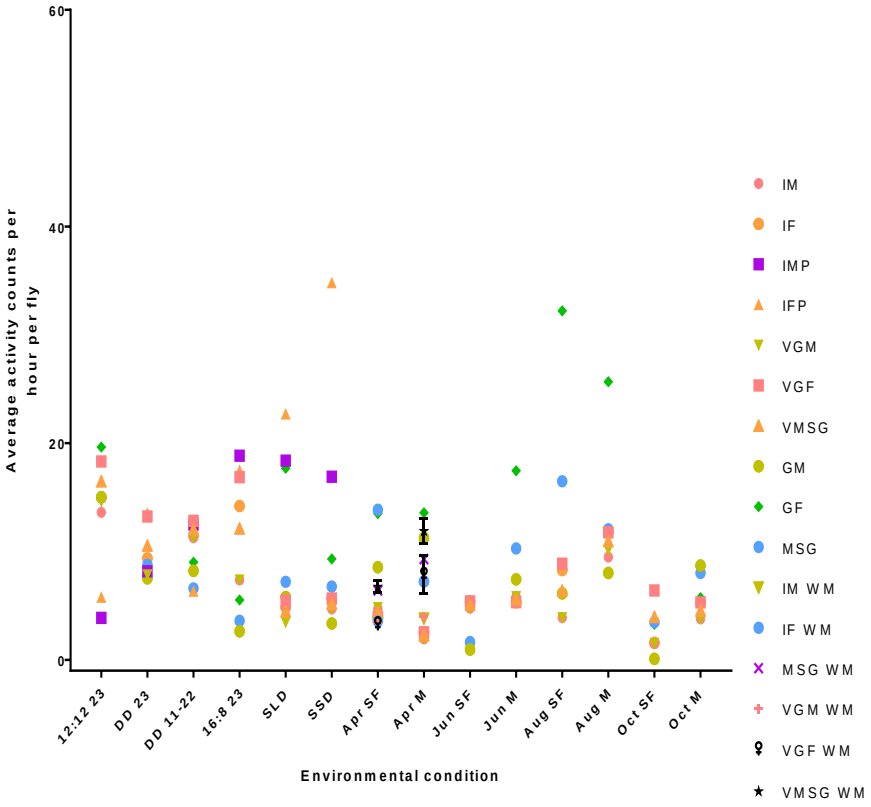


Figure 11. Mean activity per hour per fly within the various environmental conditions. Standard laboratory conditions are labelled in full. Semi-field (SF) and mimic (M) conditions are indicated after month.

Experiment 2: Can we predict oviposition rhythms in the field in laboratory based experiments?

Correlation between oviposition and adult emergence

To help decide whether direct measurement of oviposition in the laboratory would be a suitable comparative measure of emergence from field assays, the relationship of oviposition and subsequent *D. suzukii* adult emergence was examined under standard laboratory conditions (23°C 12h:12h LD). A linear relationship exhibited a high Pearson correlation coefficient (R^2=0.938) and so adult emergence could be described as a linear function of oviposition and ~82% of eggs laid developed into adults (y= 0.8234) (Figure 12). There was no significant difference in the correlation of egg to adult survival between oviposition that occurred during the light phase or in the dark phase. Thus, direct laboratory measurements of oviposition could be converted to estimated reproductive oviposition by applying a multiplication factor of 0.82.

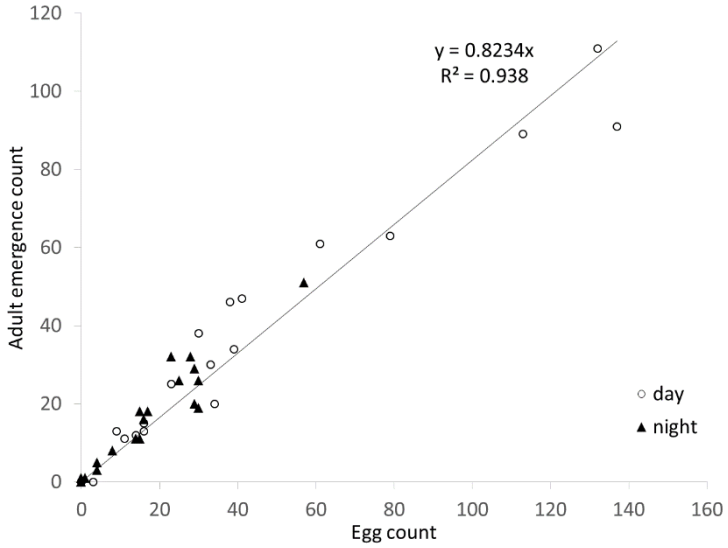


Figure 12. Correlation of the number of eggs laid and adult emergence. Flies were subjected to 12:12 L:D cycles in a constant 23°C within the reproductive oviposition confirmation assay in the laboratory. Measurements during the day are indicated by an open circle, those laid during the night indicated by a closed triangle.

Light versus dark

The average oviposition or reproductive oviposition per hour was significantly higher in the light compared to dark conditions in both laboratory and field assays. In 12:12 L:D cycle under constant 23°C 2.4-fold more eggs were laid in the light than in the dark (p<0.02)

(Figure 13a). In the 17:7 LD 17/11°C conditions eggs laid per hour in the light were 15.4-fold higher than the night (p<0.000) (Figure 13b). In the August (Figure 13c) and October (Figure 13d) field experiments, hourly reproductive oviposition was 9.1- and 25-fold higher in the day than at night, respectively (both p<0.001).

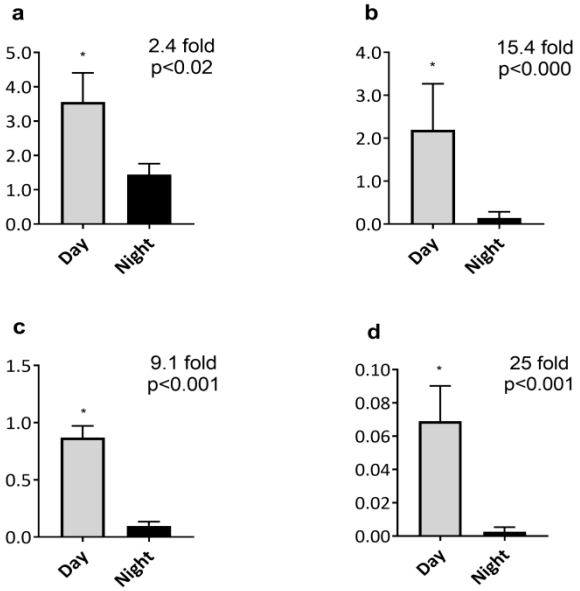


Figure 13. Average *D. suzukii* oviposition rate per hour in either the day or night. Average oviposition rates (+SE) collected under (a)12:12 L:D in 23°C, (b) 17:7 17/11°C in the laboratory or reproductive oviposition rate per hour (+SE) in either the day or night under (c) August and (d) October in the field. * indicates significant difference between day and night counts within each environmental condition.

June conditions

The June field trial was excluded from the results and analyses due to insufficient field populations which resulted in no reproductive oviposition occurring. A late frost delayed blossom and cherry development which may have also affected population build up in the orchard at this time. Although fecund females were present in the days prior to the experiment it is likely that the population density was not sufficiently high enough to detect oviposition at this time. Environmental conditions were extrapolated from the field and re-created in the laboratory as they provided additional examples of oviposition behaviour under milder 'summer' conditions.

In the June laboratory assay there were consistent peaks in oviposition from 14:00 until 20:00 on each assessment day (Figure 14). This coincided with peak temperatures between

19-22ºC. In total, 571 eggs were laid across the three days. There were significant differences in normalised eggs in relation to both time and temperature (both p<0.000) (Figure 15 a and b).

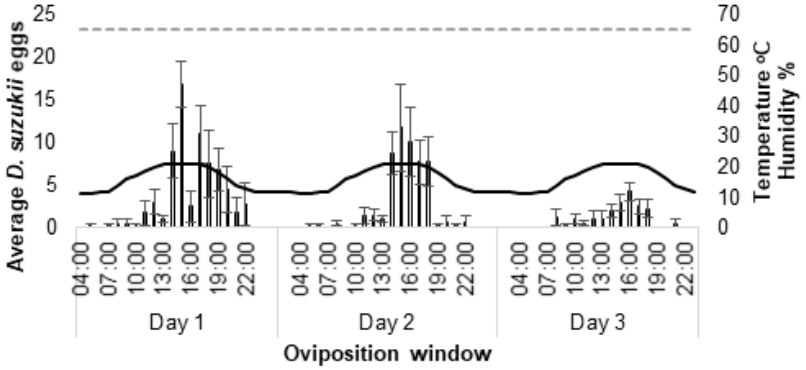


Figure 14. Average *D. suzukii* oviposition of laboratory strains under recreated June conditions within the laboratory. Average reproductive oviposition (+SE) collected in 2 hour 'oviposition windows' over three days. Average temperature (black solid line) and relative humidity (RH) (dashed grey line at x 0.5) cycled through-out. Note that relative humidity was kept constant at 65% in laboratory assays. Light is indicated by the colour of average *D. suzukii* bars. Black = darkness, grey = half-light and white = light.

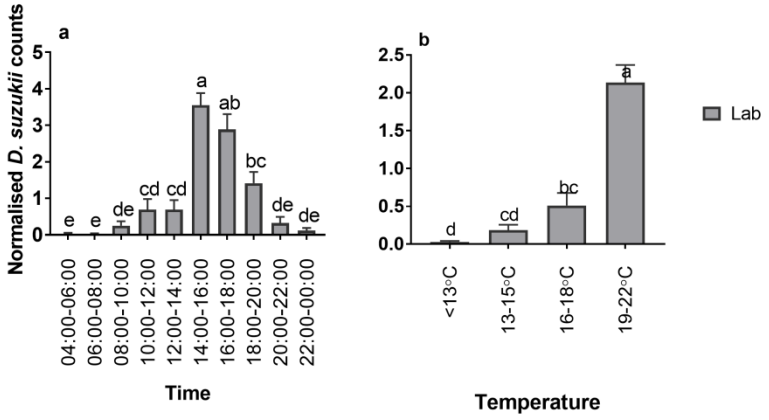


Figure 15. Normalised counts of *D. suzukii* oviposition in June conditions within the laboratory. Normalised oviposition rates (+SE) in relation to time (a) and temperature (b).

August conditions

During the August field assay, sunrise occurred at 05:24 and sunset at 20:42 on day one of the assay (2nd August 2016). The photoperiod was 15.3:8.7 h light: dark. Reproductive

oviposition occurred in two daily peaks on either side of the daily temperature warmest period (Figure 16a). The morning peak occurred 0-2 hours after sunrise on 3rd August, 4-6 hours after sunrise on 4th August and 2-4 hours after sunrise on 5th August. Evening peaks occurred 12-14 hours after sunrise (3.5-1.5 hours before sunset) on 3rd August and 14-16 hours after sunrise (3.5 hours before to 0.5 after sunset) on 4th August with no obvious peak on 2nd August. Daytime dips in reproductive oviposition occurred when temperatures exceeded 30°C.

Although there was a strong diurnal rhythm in reproductive oviposition during the August field assay (see Figure 13c), no significant differences in normalised reproductive oviposition were found between the different time intervals during day light (Figure 17a). Nevertheless, there was a significant effect of temperature on normalised reproductive oviposition during day-light hours with highest normalised oviposition occurring between 27-30 ºC ($p<0.006$) (d).

When August conditions were re-created in the laboratory, overall egg laying was low (49 in total) although egg laying did occur on each day of assessment (Figure 16b). There was a significant effect of time interval on normalised oviposition ($p<0.011$), (Figure 17b) with fewer eggs laid between 05:00-07:00, 09:00-11:00, 11:00-13:00 and 13:00-15:00. There was higher egg laying in temperatures between 19-22 ºC although this was not significant (Figure 17e).

In the laboratory, peaks and troughs of *D. suzukii* oviposition, correlated with reproductive oviposition rates in the field. There was no significant difference between the laboratory and field counts in relation to time (Figure 17c). There was also no significant difference in counts between laboratory and field assays' dependent on temperature ranges (Figure 17f). Regarding the apparent differences in the impact of daily time and temperature on day-time (reproductive) oviposition it should be noted that field temperature and humidity conditions were somewhat variable from day to day and that field temperatures were measured at the site of oviposition, which might have been visited only briefly by the flies.

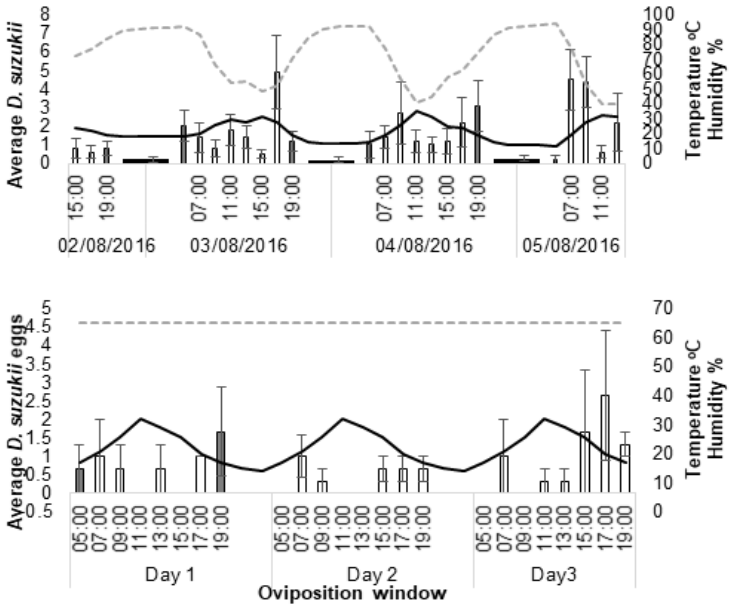


Figure 16. Average *D. suzukii* oviposition of wild and laboratory strains under August conditions within the field and laboratory. Average reproductive oviposition (+SE) of (a) wild *D. suzukii* in the field and (b) average oviposition (+SE) of laboratory strains in recreated August conditions within the laboratory (bars) in 2 hour 'oviposition windows' over three days. Average temperature (black solid line) and relative humidity (RH) (dashed grey line at x 0.5) cycled through-out. Note that relative humidity was kept constant at 65% in laboratory assays. Light is indicated by the colour of average *D. suzukii* bars. Black = darkness, grey = half-light and white = light.

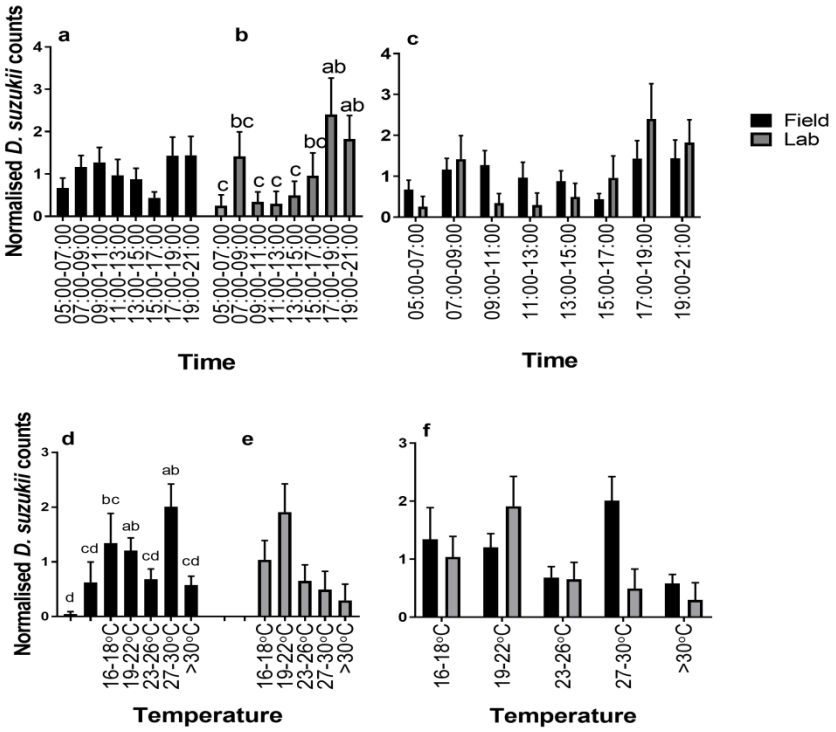


Figure 17. Normalised *D. suzukii* oviposition under August conditions in relation to time and temperature. Normalised counts of *D. suzukii* reproductive oviposition from (a) field assay (+SE) (black bars) and (b) laboratory oviposition (+SE) (grey bars) in August conditions and (c) field vs. laboratory (+SE) in relation to time. Normalised counts of *D. suzukii* reproductive oviposition from (d) field assay (+SE) and (e) laboratory oviposition (+SE) in August conditions and (f) field vs. laboratory (+SE) in relation to temperature. Different letters identify significant differences between time points within each setting.

October conditions

In the October field assay, sunrise was at 07:04 and sunset at 18:28 on day one of the assay (5th October 2016). The photoperiod was 11.4:12.6 L:D. Peaks in reproductive oviposition occurred 6-8 hours after sunrise on 5th October (6-4 hours before sunset) and 8-10 hours after sunrise on 6th October (4-2 hours before sunset) (Figure 18a). There was no obvious peak on the 7th October. Overall reproductive oviposition was low (25 in total) at this time in the field. Peaks in reproductive oviposition appeared to occur at peak temperature each day. There was a significant effect of time on normalised reproductive oviposition counts (p<0.039) (Figure 19a) with no reproductive oviposition occurring between 08:45-10:45 and 16:45-18:45 on any day. There was no significant effect of temperature on normalised reproductive oviposition (Figure 19d).

In the laboratory under the October conditions, oviposition occurred throughout each day with no specific peaks identifiable (Figure 18b). Overall 146 eggs were laid in total across the three days. There was no significant effect of time or temperature on normalised counts (Figure 19b and e).

There were significant differences between the laboratory and field assays with October conditions at only two time points; 08:45-10:45 (p=0.03) and 16:45-18:45 (p= 0.002). During these periods no eggs were laid in the field (Figure 19c). There were also significant differences in egg counts in 2 of the 4 temperature ranges (Figure 19f). At 13-15°C, counts in reproductive oviposition in the field were significantly lower than oviposition in the laboratory (p=0.05). However, at 16-18°C, counts in reproductive oviposition in the field were significantly higher than in the laboratory (p=0.02).

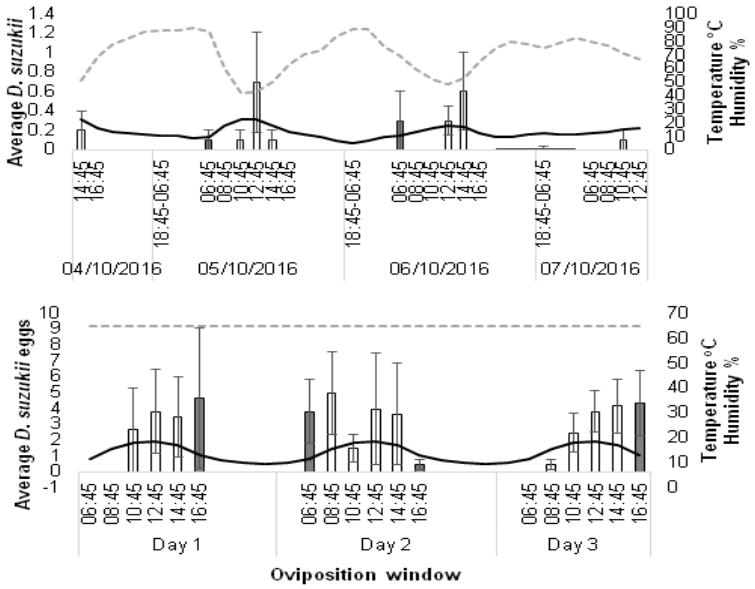


Figure 18. Average *D. suzukii* oviposition of wild and laboratory strains under October conditions within the field and laboratory. Average reproductive oviposition (+SE) of (a) wild *D. suzukii* in the field and (b) average oviposition (+SE) of laboratory strains in recreated October conditions within the laboratory (bars) in 2 hour 'oviposition windows' over three days. Average temperature (black solid line) and relative humidity (RH) (dashed grey line at x 0.5) cycled through-out. Note that relative humidity was kept constant at 65% in laboratory assays. Light is indicated by the colour of average *D. suzukii* bars. Black = darkness, grey = half-light and white = light.

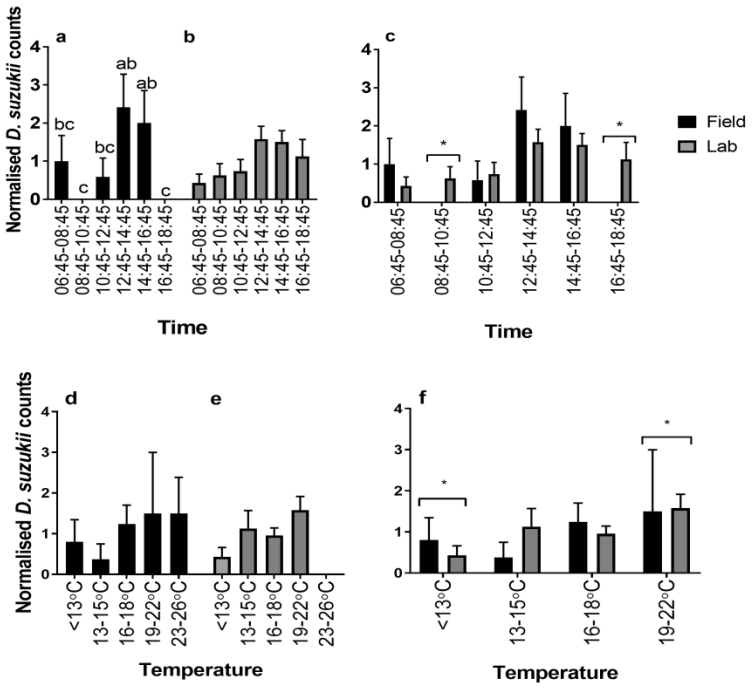


Figure 19. Normalised *D. suzukii* oviposition under October conditions in relation to time and temperature. Normalised counts of *D. suzukii* reproductive oviposition from (a) field assay (+SE) (black bars) and (b) laboratory oviposition (+SE) (grey bars) in October conditions and (c) field vs. laboratory (+SE) in relation to time. Normalised counts of *D. suzukii* reproductive oviposition from (d) field assay (+SE) and (e) laboratory oviposition (+SE) in August conditions and (f) field vs. laboratory (+SE) in relation to temperature. Different letters identify significant differences between time points within each setting.

Locomotion activity and oviposition

Locomotion activity profiles were collected under the same recreated temperature and light cycles used in the laboratory oviposition assay, obtained from the field. Notably, times of peak locomotor activity coincided with peak oviposition for the June (Figure 20a) and October (Figure 20c), but not the August condition (Figure 20b). Further, oviposition rates at dawn in August and during the entire morning in June were lower than might be expected based on the accompanying locomotor activity levels. Generally, the best coherence between relative locomotor and oviposition activity levels was observed for the mid-afternoon to dusk period.

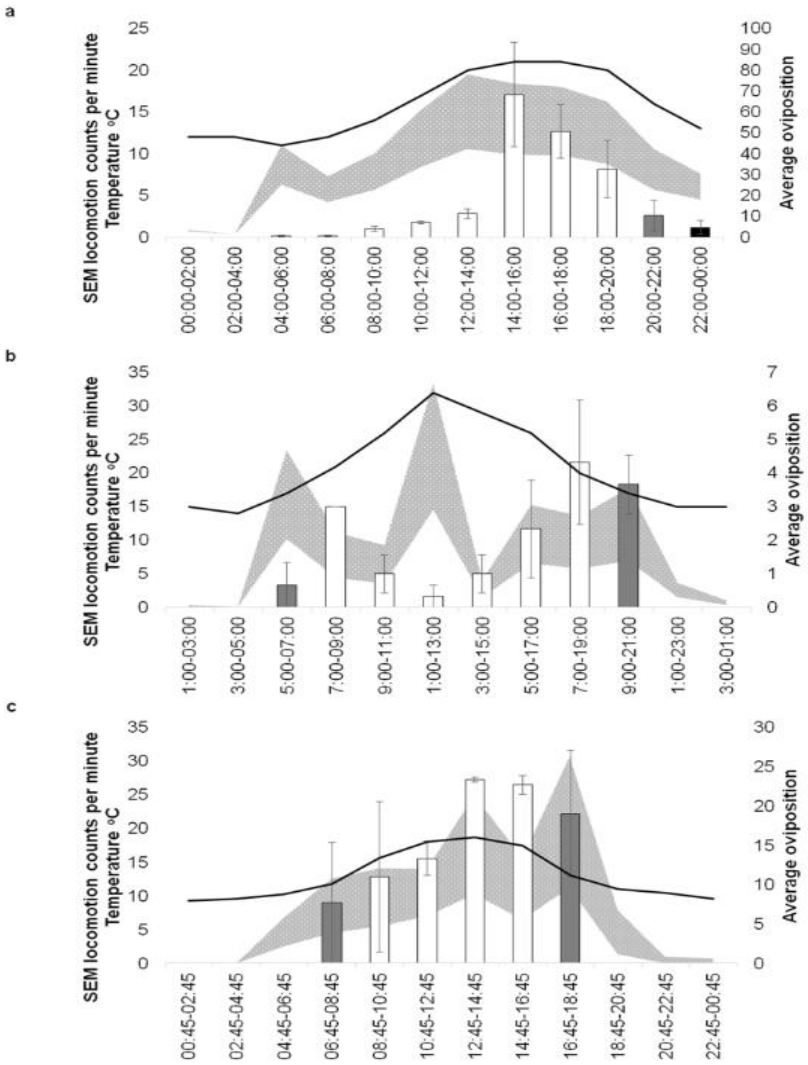


Figure 20. Mean locomotion counts/minute of mix sex groups in relation to average oviposition. Standard error of mean range of locomotion (counts per minute) of mix sex groups (grey area) in relation to average oviposition (+SE) (bars) and temperature (dash grey line) in the laboratory. Collected under recreated (a) June, (b) August and (c) October conditions. Light is indicated by the colour of average counts bars: Black in darkness, grey in half-light and white in full-light. Oviposition was assessed during the light phase (04:00-00:00 under June conditions, 05:00-21:00 under August and 06:45-18:45 under October).

Experiment 3: How will the presence of another species affect oviposition behaviour in D. suzukii?

Next generation emergence

Significantly more *D. suzukii* adults emerged from blank media that contained no eggs initially than media pre-inoculated with *D. melanogaster* (Figure 21) (Experiment 1, $F_{1,8}$ = 8.06; P = 0.022. Experiment 2, $F_{1,10}$ = 159.26; P = < 0.001. Experiment 3, $F_{1,10}$ = 14.66; P = 0.03). However, we cannot confrim whether this was due to fewer eggs being laid initally or if egg to adult survival was lower. It had been suggested that this could be the results of cannibalistic tendencies of some *Drosophila* species which occurs when nutrition is restricted (Ahmad et al., 2015). Morphological defects can be a visual indication that cultures have been maintained on diets lacking nutrition (Vijendravarma et al., 2010). Although no quantitative measurements were taken, there was no noticeable reduction in body and wing size in our competition experiment to indicate diet restriction which promotes cannibalism. However it has been found that a 'basal level of cannibalism' does occur in *D. melanogaster* cultured on a standard yeast/sugar diet (Bhattacharyya, 2015b, Bhattacharyya, 2015a). In *D. melanogaster* the younger mobile larval stages hunt immobile larvae preparing to pupate (Vijendravarma et al., 2013). We would therefore expect to see a reduction in emergence in the first inoculation species and not the second. This would not explain why *D. suzukii* emergence was lower from media pre-inoculated with *D. melanogaster*.

There was no significant difference between the numbers of *D. melanogaster* that emerged from blank media or the *D. suzukii* pre-inoculated media (Figure 22). As with many native *Drosophila* species, *D. melanogaster* utilise damaged and decomposing fruit for egg laying and may not perceive the presence of another species as detrimental. Other groups have also found *D. suzukii* oviposition wounds expose fruit that would have otherwise been immune to other *Drosophila* specie searching for egg laying sites.

There was no significance between the numbers of *D. suzukii* that emerged from blank media compared to media that had previously been exposed to other *D. suzukii* (Figure 23). If significantly more *D. suzukii* emerged from the pre-inoculated media, it could have indicated oviposition aggregation pheromones as found in some *Drosophila* species (Wertheim, 2001, Symonds and Wertheim, 2005). However, in wild populations, if given a choice, *D. suzukii* eggs are typically deposited either singly into fruit or in very small

clutches (Mitsui et al., 2006) indicating *D. suzukii* preference for oviposition sites low in egg counts or free from both *D. melanogaster* and conspecifics.

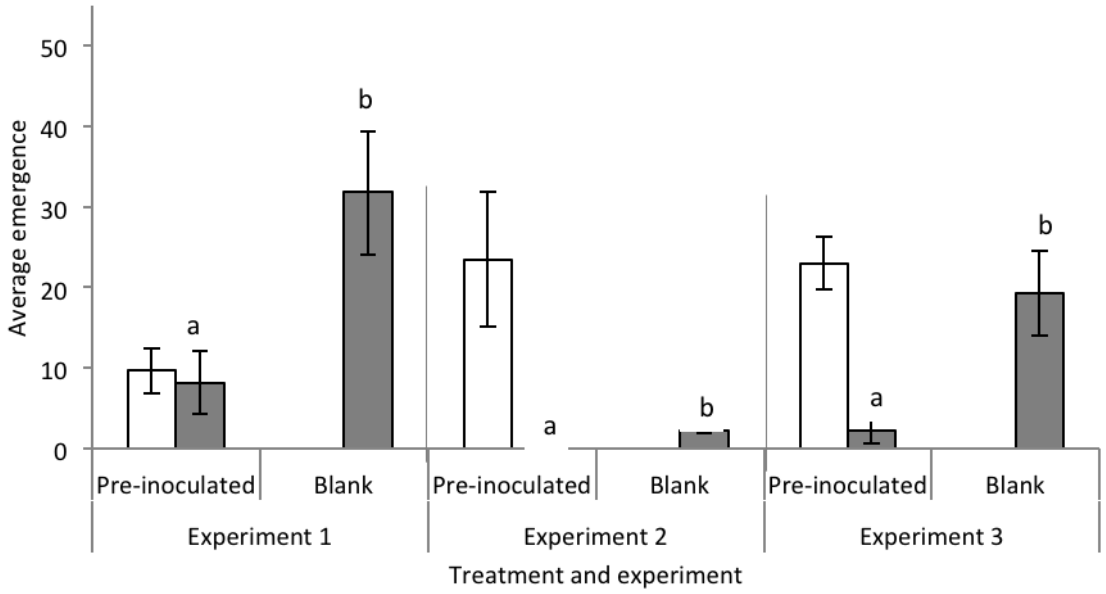


Figure 21. Average emergence (±S.E.) of *D. suzukii* adult offspring indicated by grey bars, from either cornmeal media (experiment 1) or raspberry fruits (experiment 2 and 3) pre-inoculated with *D. melanogaster* eggs or blank media. White bars display emergence of *D. melanogaster* pre-inoculation. Different lower case letters indicate significant difference between average *D. suzukii* emergence from inoculated and blank media for each experiment.

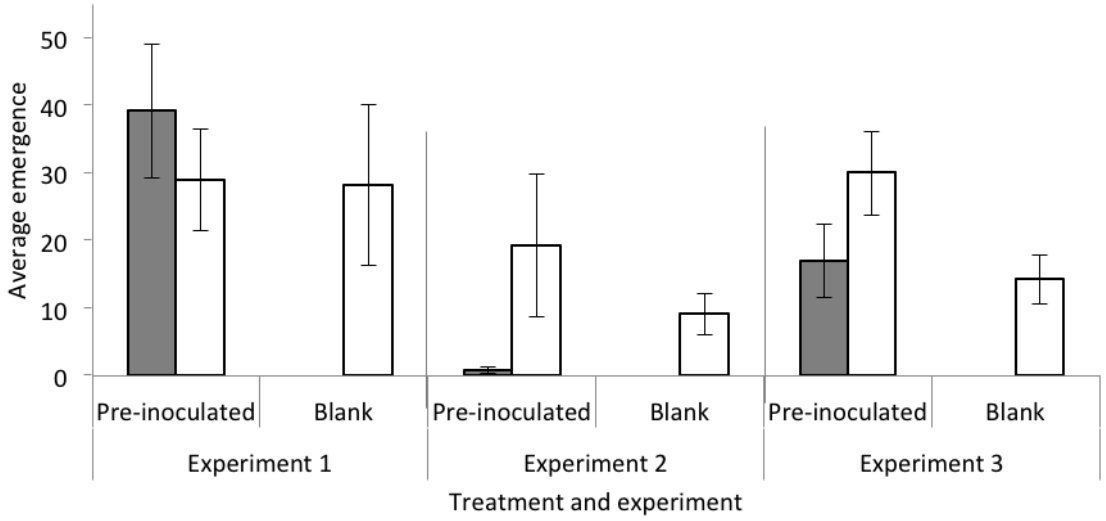


Figure 22. Average emergence (±S.E.) of *D. melanogaster* adult offspring indicated by white bars, from either cornmeal media (experiment 1) or raspberry fruits (experiment 2 and 3) pre-inoculated with *D. suzukii* eggs or blank media. Grey bars display emergence *D. suzukii* of pre-inoculation. NSD between treatments in average *D. melanogaster* emergence from inoculated and blank media for each experiment.

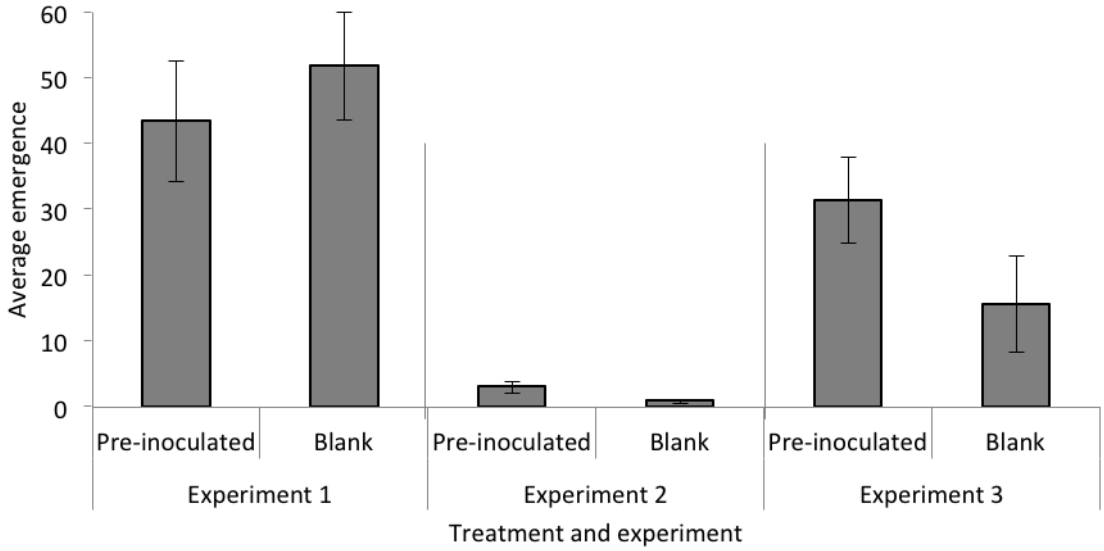


Figure 23. Average emergence (±S.E.) of *D. suzukii* adult offspring from either cornmeal media (experiment 1) or raspberry fruits (experiment 2 and 3) pre-inoculated with *D. suzukii* eggs or blank media. NSD between treatments in emergence from inoculated and blank media for each experiment

Female oviposition choice

Significantly more *D. suzukii* eggs were counted in blank medium (mean 24 eggs) than in medium pre-inoculated by *D. melanogaster* (average 3 eggs) in the female oviposition choice experiment (Figure 24) (t9 =−3.122; P =0.012).

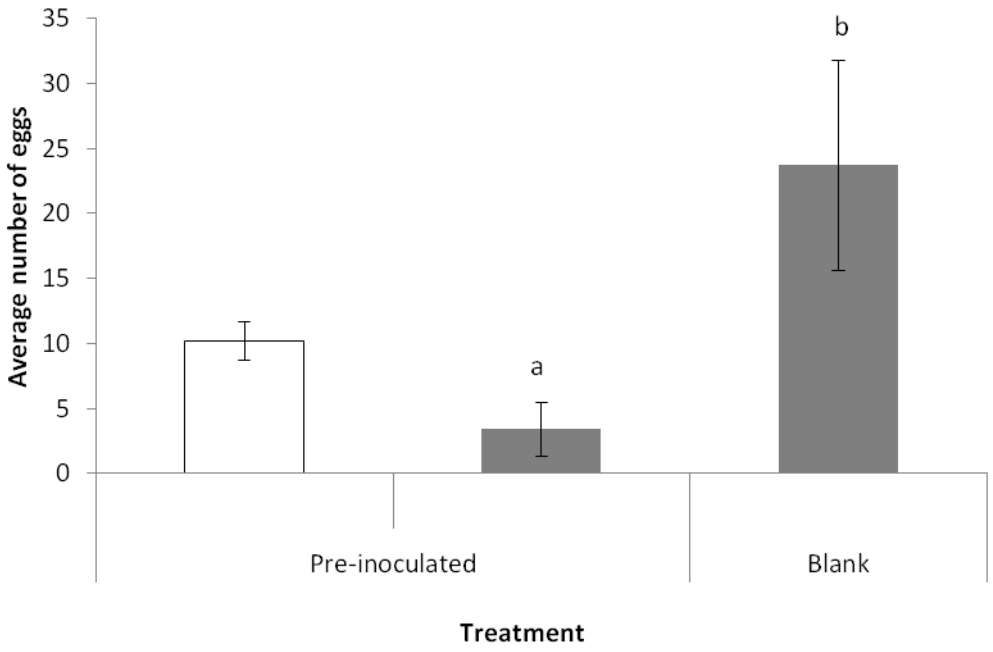


Figure 24. Number of *D. suzukii* eggs laid in substrates pre- inoculated by *D. melanogaster* or blank in competition assay.Mean number (±S.E.) of *D. suzukii* eggs laid in either blank or pre-inoculated media indicated by grey bars. White bars indicate average number of *D. melanogaster* eggs in the pre-inoculation treatment.

Experiment 4: Is there any impact of the circadian clock on plant protection product susceptibility?

LC50

The LC50 values were identified for each pesticide. The LC50 was also identified for specific points after application which did not appear to change over time (Table 4).

Table 4. LC50 values of each plant protection product treatment, over time, on *D. suzukii*, presented as percent of recommended field rate.

Treatment	24 h	48 h	72 h	96 h	168 h
Cyantraniliprole	5.5	4.8	4.7	4.5	3.9
Lambda-cyhalothrin	7.9	7.7	8.2	8.2	8.2
Pyrethrum	2.2	2.2	2.0	2.0	2.1
Spinosad	15.5	13.7	13.1	12.7	11.3

Dose response: mortality

For cyantraniliprole there were significant differences in mortality between doses at each time point after application (Table 5, Figure 25). No difference in mortality was detected in flies treated with 0.3-1.5% FR in comparison to the control on any assessment day after treatment application. There was also no difference in mortality between the control and 3% of FR from 24-96 h after application but a difference did occur at 168 h. Survival was reduced in flies treated with 6-50% FR in comparison to the control 24-168 h after application. Twenty-four hours after spray application, only 7% survival of flies treated with 50% FR ensued. This gradually declined to 1% after 168 h.

Table 5. Differences in mortality of *D. suzukii* adults treated with dilutions of field rate cyantraniliprole, overall, and in pairwise comparisons to a distilled water control, over time, after application. NSD indicates no significant difference.

	Time (hours)				
	24	**48**	**72**	**96**	**168**
***p* value**	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$
***F* value**	$F_{8,45}=24.54$	$F_{8,45}=21.53$	$F_{8,45}=25.28$	$F_{8,45}=19.88$	$F_{8,45}=20.83$
Dose	**pairwise interactions with control (*p* value)**				
0.33%	NSD	NSD	NSD	NSD	NSD
0.75%	NSD	NSD	NSD	NSD	NSD
1.50%	NSD	NSD	NSD	NSD	NSD
3%	NSD	NSD	NSD	NSD	0.040
6%	0.000	0.000	0.000	0.000	0.000
12%	0.000	0.000	0.000	0.000	0.000
25%	0.000	0.000	0.000	0.000	0.000
50%	0.000	0.000	0.000	0.000	0.000

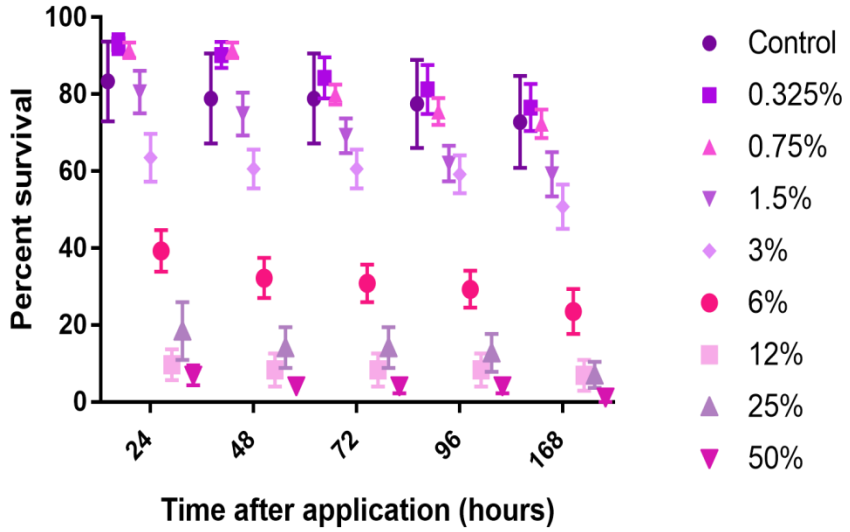


Figure 25. Mortality response of *D. suzukii* adults to dilutions of field rate cyantraniliprole, over time, after application.

A significant reduction in survival of flies was observed at all doses, of lambda-cyhalothrin in comparison, to the control, 24-168 h after application (Table 6, Figure 26). After 24 h, there was no survival of *D. suzukii* treated with 100% FR and only 1% survival in flies treated with 50% FR. Survival did not change over time at any of the doses.

Table 6. Differences in mortality of *D. suzukii* adults treated with dilutions of field rate lambda-cyhalothrin, overall, and in pairwise comparisons to a distilled water control, over time, after application. NSD indicates no significant difference.

	Time (hours)				
	24	**48**	**72**	**96**	**168**
***p* value**	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$
***F* value**	$F_{5,42}=41.08$	$F_{5,42}=37.47$	$F_{5,42}=33.44$	$F_{5,42}=30.71$	$F_{5,42}=32.18$
Dose	**pairwise interactions with control (*p* value)**				
6%	0.002	0.003	0.008	0.009	0.007
12%	0.000	0.000	0.000	0.000	0.000
25%	0.000	0.000	0.000	0.000	0.000
50%	0.000	0.000	0.000	0.000	0.000
100%	*	*	*	*	*

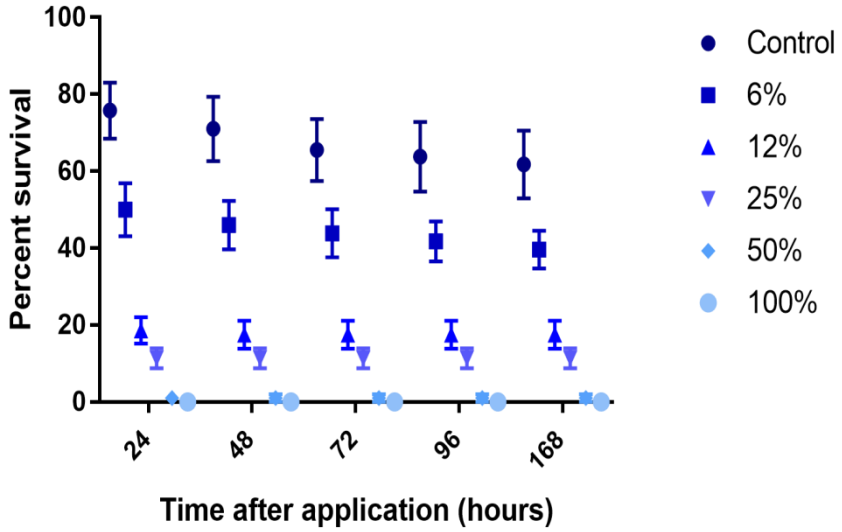


Figure 26. Mortality response of *D. suzukii* adults to dilutions of field rate lambda-cyhalothrin, over time, after application.

For pyrethrum, there was a significant reduction in survival in all doses 24 h after application in comparison to the control (Table 7, Figure 27). No survival occurred after 24 h in 25% FR; the highest dose applied. There was a 3% survival after 24 h in flies treated with 12% FR, which did not decrease over time. Survival did not change in any of the doses, over time, after application.

Table 7. Differences in mortality of *D. suzukii* adults treated with dilutions of field rate pyrethrum, overall, and in pairwise comparisons to a distilled water control, over time, after application. NSD indicates no significant difference.

	Time				
	24	**48**	**72**	**96**	**168**
***p* value**	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$
F	$F_{5,30}=16.89$	$F_{5,30}=17.01$	$F_{5,30}=16.40$	$F_{5,30}=16.50$	$F_{5,30}=16.49$
Dose	**pairwise interactions with control (*p* value)**				
1.5%	0.031	0.029	0.017	0.019	0.026
3%	0.000	0.000	0.000	0.000	0.000
6%	0.003	0.002	0.002	0.002	0.002
12%	0.000	0.000	0.000	0.000	0.000
25%	*	*	*	*	*

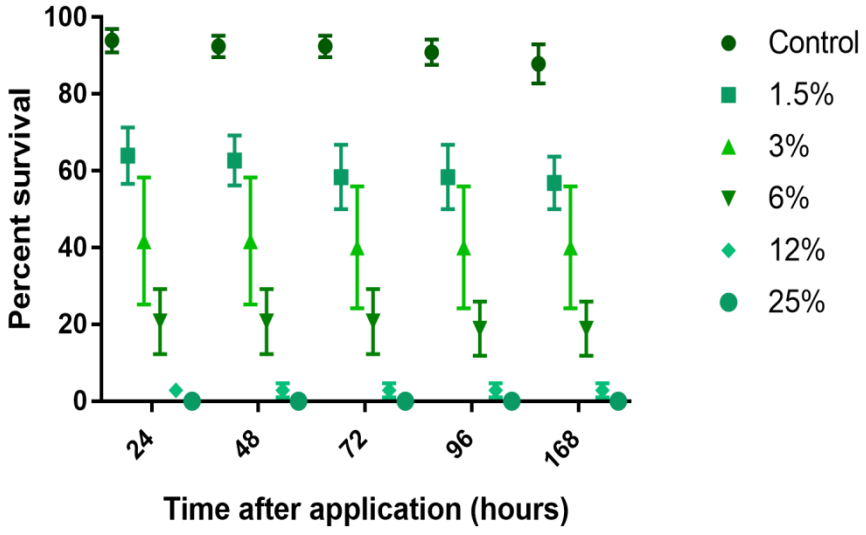


Figure 27. Mortality response of *D. suzukii* adults to dilutions of field rate pyrethrum, over time, after application.

There was no significant difference in survival between the control and flies treated with 6% FR of spinosad from 24-168 h after application (Table 8, Figure 28). A reduction in survival between 12-100% FR did occur in comparison with the control, 24 h after application. Twenty-four hours after treatment application, an average of 7% *D. suzukii* survived 100% FR for spinosad which fell to 3% 168 h after application. After 24 h, 17% survival occurred in flies treated with 50% FR, which fell to 13% after 168 h. There was no significant reduction in survival in any of the doses over time after application.

Table 8. Differences in mortality of *D. suzukii* adults treated with dilutions of field rate spinosad, overall, and in pairwise comparison to a distilled water control, over time, after application. NSD indicates no significant difference.

	Time				
	24	**48**	**72**	**96**	**168**
***p* value**	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$
F	$F_{5,30}=13.65$	$F_{5,30}=12.25$	$F_{5,30}=12.04$	$F_{5,30}=12.07$	$F_{5,30}=11.86$
Dose	**pairwise interactions with control (*p* value)**				
6%	NSD	NSD	NSD	NSD	NSD
12%	0.002	0.002	0.002	0.002	0.001
25%	0.002	0.003	0.004	0.003	0.002
50%	0.000	0.001	0.001	0.001	0.001
100%	0.000	0.000	0.001	0.000	0.001

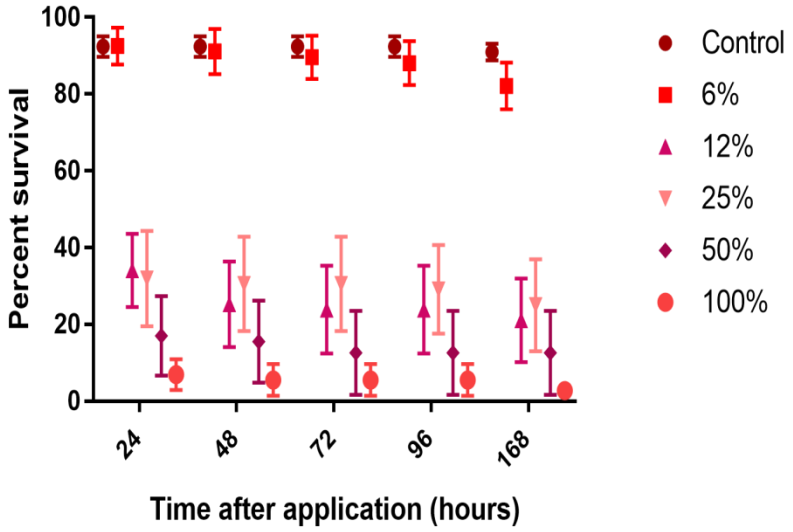


Figure 28. Mortality response of *D. suzukii* adults to dilutions of field rate spinosad, over time, after application.

Dose response: oviposition

The number of eggs laid by *D. suzukii* varied for all pesticides in relation to dose and time after spray application.

Significantly more eggs were laid in the control than by adult flies treated with 12-50% FR cyantraniliprole, 24-168 h after application (Table 9a, Figure 29a). More eggs were laid in the control than 3 and 6% FR 24-72 h after application. There was no difference in the total numbers of eggs laid in the control and 0.75 and 1.5% FR from 24-168 h after application. More eggs were laid in total in 0.33% treated flies than the control 24, 96 and 168 h after application.

Oviposition per female was also influenced by cyantraniliprole concentration (Table 9b, Figure 29b). Twenty-four hours after application, doses between 3-50% FR reduced the numbers of eggs per live female (EPF) in comparison to the control. Forty-eight hours after application, a reduction in EPF in 6, 12 and 25% FR occurred in comparison to the control and 3, 6 and 25% FR 72 hours after application. No reduction in EPF was observed between the control and 0.75 and 1.5% FR at any point after application. There was no difference in EPF when treated with 50% FR from 48-168 h after application and an increase occurred in EPF when treated with 0.33% FR 24 and 168 h after application in comparison to the control.

Table 9. Table of overall significance and pairwise interactions of (a) total number of eggs and (b) number of *D. suzukii* eggs per female, in each dose treated with cyantraniliprole, over time, in comparison to the distilled water control and pairwise interactions. NSD indicates no significant difference. *Overall significance due to interactions between 0.325% and 1.5 and 3% FR.

a

	Time (hours)				
	24	**48**	**72**	**96**	**168**
***p* value**	0.000	0.000	0.000	0.000	0.000
Dose	**pairwise interactions with control (*p* value)**				
0.33%	0.001	NSD	NSD	0.046	0.031
0.75%	NSD	NSD	NSD	NSD	NSD
1.50%	NSD	NSD	NSD	NSD	NSD
3%	0.005	0.036	0.009	NSD	NSD
6%	0.001	0.001	0.000	NSD	NSD
12%	0.001	0.000	0.000	0.000	0.001
25%	0.001	0.000	0.000	0.000	0.000
50%	0.011	0.000	0.000	0.000	0.000

b

	Time (hours)				
	24	**48**	**72**	**96**	**168**
***p* value**	0.000	0.023	0.007	0.048*	NSD
Dose	**pairwise interactions with control (*p* value)**				
0.33%	0.003	NSD	NSD	NSD	0.035
0.75%	NSD	NSD	NSD	NSD	NSD
1.50%	NSD	NSD	NSD	NSD	NSD
3%	0.006	NSD	0.008	NSD	NSD
6%	0.001	0.016	0.001	NSD	NSD
12%	0.001	0.032	NSD	NSD	NSD
25%	0.001	0.049	0.004	NSD	NSD
50%	0.01	NSD	NSD	NSD	NSD

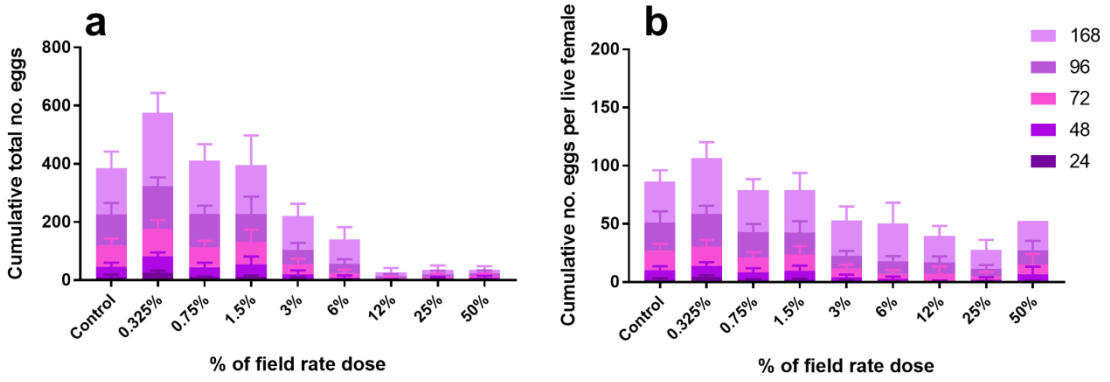


Figure 29. (a) Cumulative number of total eggs over time and (b) cumulative eggs per live *D. suzukii* female treated with cyantraniliprole at dilutions of the field rate.

For Lambda-cyhalothrin, significantly fewer eggs were laid from 24-168 h after application in treatments which were 12% FR and above in comparison to the control (Table 10a, Figure 30a). Fewer eggs were also laid in the 6% FR treatment 24, 72 and 96 h after application.

A significant reduction in the number of eggs per live female (EPF) laid 24 h after application occurred (Table 10b, Figure 30b) with fewer eggs laid in all treatments in comparison to the control. There was no significant difference in the EPF between the control and 6-50% FR from 48 h onwards. No eggs were laid in the 100% FR from 48 h onwards due to 100% mortality in parent flies at the 24 h assessment.

Table 10. Table of overall significance and pairwise interactions of (a) total number of eggs and (b) number of *D. suzukii* eggs per female, in each dose treated with lambda-cyhalothrin, over time, in comparison to the distilled water control and pairwise interactions. NSD indicates no significant difference.

a

	Time (hours)				
	24	**48**	**72**	**96**	**168**
***p* value**	0.000	0.000	0.000	0.000	0.000

Dose	**pairwise interactions with control (*p* value)**				
6%	0.001	NSD	0.023	0.005	NSD
12%	0.000	0.000	0.001	0.000	0.022
25%	0.000	0.000	0.000	0.000	0.002
50%	0.000	0.000	0.000	0.000	0.000
100%	0.000				

b

	Time (hours)				
	24	**48**	**72**	**96**	**168**
***p* value**	0.00	NSD	NSD	NSD	NSD

Dose	**pairwise interactions with control (*p* value)**				
6%	0.000	NSD	NSD	NSD	NSD
12%	0.000	NSD	NSD	NSD	NSD
25%	0.000	NSD	NSD	NSD	NSD
50%	0.000	NSD	NSD	NSD	NSD
100%	0.000				

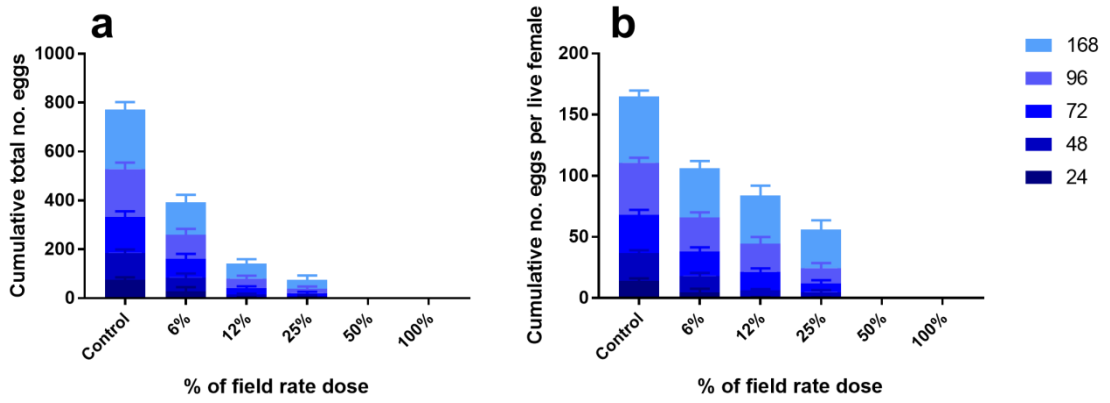


Figure 30. (a) Cumulative number of total eggs over time and (b) cumulative eggs per live *D. suzukii* female treated with lambda-cyhyalothrin at dilutions of the field rate.

Significantly fewer eggs were laid, in total, in 6-25% FR pyrethrum from 24-168 h after application in comparison to the control (Table 11a, Figure 31a). There was also a significant reduction in total number of eggs in the 3% FR treatment in comparison to the control from 48-168 h after application.

Significantly fewer EPF were laid in 6-25% FR treated samples, in comparison to the control, 24 h after application (Table 11b, Figure 31b) but no difference occurred between the control and 6-12% FR from 48 h onwards. No oviposition occurred in 25% FR treatment from 48 h onwards due to 100% mortality of parent flies at the 24 h assessment.

Table 11. Table of overall significance and pairwise interactions of (a) total number of eggs and (b) number of *D. suzukii* eggs per female, in each dose treated with pyrethrum, over time, in comparison to the distilled water control and pairwise interactions. NSD indicates no significant difference.

a

	Time (hours)				
	24	**48**	**72**	**96**	**168**
***p* value**	0.003	0.000	0.000	0.000	0.000

Dose	**pairwise interactions with control (*p* value)**				
1.5%	NSD	NSD	NSD	NSD	NSD
3%	NSD	0.027	0.039	0.005	0.006
6%	0.002	0.001	0.001	0.000	0.000
12%	0.009	0.000	0.000	0.000	0.000
25%	0.002				

b

	Time (hours)				
	24	**48**	**72**	**96**	**168**
***p* value**	0.01	NSD	NSD	NSD	NSD

Dose	**pairwise interactions with control (*p* value)**				
1.5%	NSD	NSD	NSD	NSD	NSD
3%	NSD	NSD	NSD	NSD	NSD
6%	0.005	NSD	NSD	NSD	NSD
12%	0.020	NSD	NSD	NSD	NSD
25%	0.006				

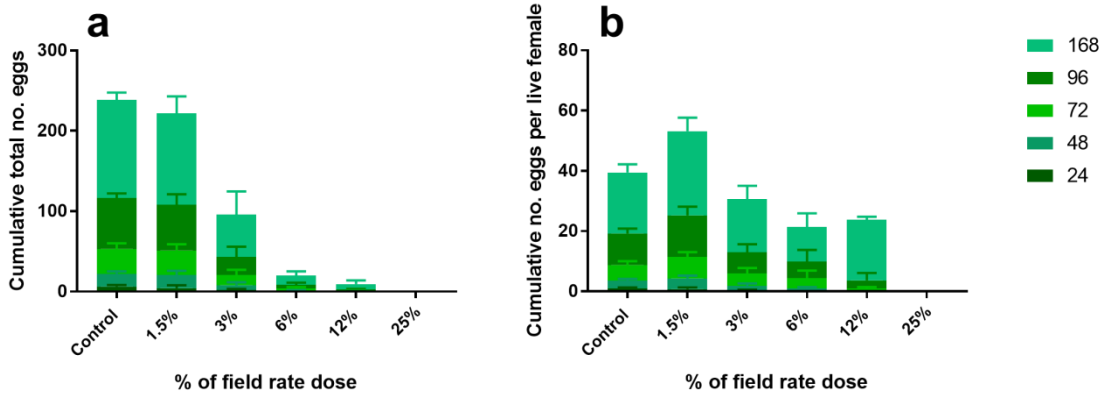


Figure 31. (a) Cumulative number of total eggs over time and (b) cumulative eggs per live *D. suzukii* female treated with pyrethrum at dilutions of the field rate.

The total number of eggs was significantly lower in *D. suzukii* treated with 12-100% FR spinosad from 24-168 h after application (Table 12a, Figure 32a) and at 48 and 168 h after application with 6% FR in comparison to the control. There was a reduction in EPF at 24 and 96 h after application (Table 12b, Figure 32b). Twenty-four hours after application fewer EPF were laid in treatments 12-100% FR in comparison to the control, but no difference in EPF between the control and treatments at 48, 72 and 168 h after application occurred. A decrease in EPF occurred in samples 6-25% FR in comparison with the control. However,

no difference in EPF occurred between samples treated with 50 and 100% FR and the control from 48 h after application onwards.

Table 12. Table of overall significance and pairwise interactions of (a) total number of eggs and (b) number of *D. suzukii* eggs per female in each dose treated with spinosad, over time, in comparison to the distilled water control and pairwise interactions. NSD indicates no significant difference.

	Time (hours)				
	24	**48**	**72**	**96**	**168**
***p* value**	0.002	0.000	0.001	0.000	0.002
Dose	**pairwise interactions with control (*p* value)**				
6%	NSD	0.018	NSD	NSD	0.005
12%	0.014	0.000	0.001	0.001	0.004
25%	0.011	0.002	0.028	0.001	0.010
50%	0.000	0.000	0.001	0.000	0.001
100%	0.001	0.000	0.000	0.000	0.000

b

	Time (hours)				
	24	**48**	**72**	**96**	**168**
***p* value**	0.001	NSD	NSD	0.03	0.05
Dose	**pairwise interactions with control (*p* value)**				
6%	NSD	NSD	NSD	0.016	0.003
12%	0.010	NSD	NSD	0.049	NSD
25%	0.013	NSD	NSD	0.006	NSD
50%	0.000	NSD	NSD	NSD	NSD
100%	0.001	NSD	NSD	NSD	NSD

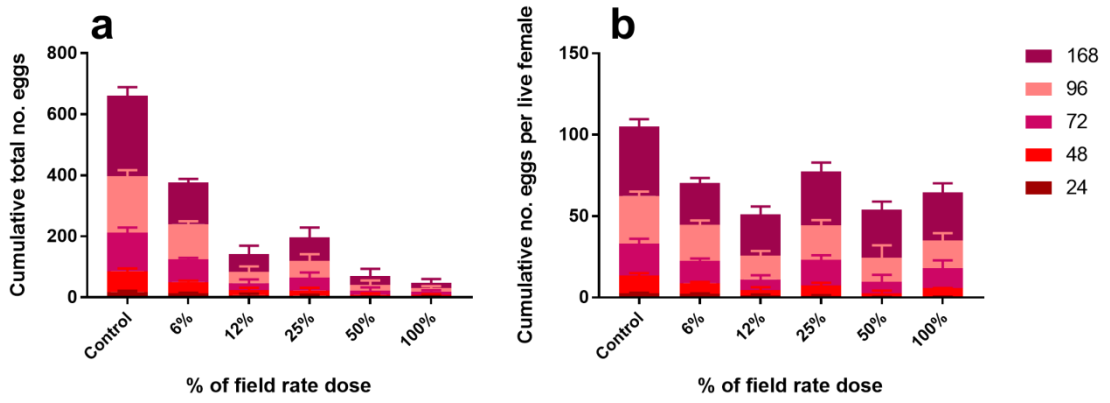


Figure 32.(a) Cumulative number of total eggs over time and (b) cumulative eggs per live *D. suzukii* female treated with spinosad at dilutions of the field rate.

Dose response: transgenerational impacts on survival

For cyantraniliprole and pyrethrum no significant reduction in survival of eggs through to adult emergence occurred at any of the doses in comparison to the distilled water control at any time point (data not shown).

For lambda-cyhalothrin, there was a significant difference in egg to pupa (p<0.006, $F_{4,22}$=4.79), pupa to adult (p<0.001, $F_{3,17}$=12.39) and egg to adult (p<0.043, $F_{4,22}$=2.95) survival 24 h after treatment application in comparison to the control (Figure 33a). A reduction in the survival of offspring arose in samples treated with 6% FR in egg to pupa (p<0.001, t_{22}=3.87), egg to adult (p<0.007, t_{22}=3.00) and in pupa to adult (p<0.000, t_{17}=-14.76) survival in relation to the control.

Forty-eight hours after application pupa to adult emergence was reduced (p<0.014, $F_{3,20}$=4.53) within the 6% FR (p<0.045, t_{20}=2.14) compared to the distilled water control (Figure 33b).

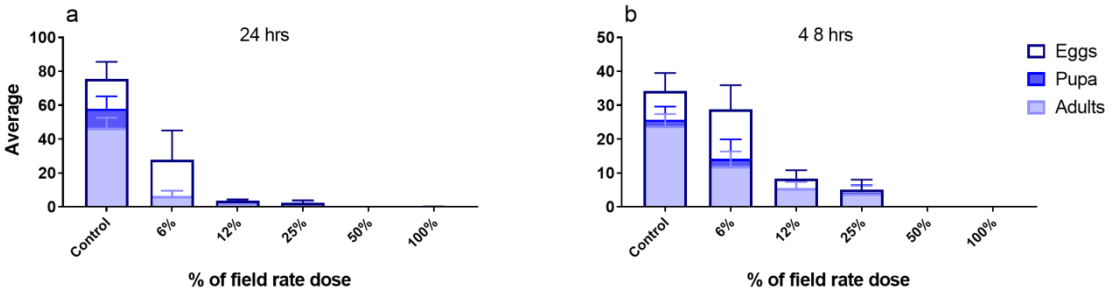


Figure 33. Survival of *D. suzukii* offspring life stages (a) 24 h and (b) 48 h after parents were treated with lambda-cyhalothrin dilutions of field rate (75ml/ha).

For spinosad, a reduction in development from eggs to pupa (p<0.021, $F_{5,26}$=3.24), and egg to adult survival (p<0.011, $F_{5,26}$=3.75) 24 h after treatment application was observed. Egg to pupa (p<0.04, t_{26}=2.16) and egg to adult (p<0.027, t_{26}=2.34) survival was reduced at 6% FR compared to the control (Figure 34a).

There was a significant difference in egg to pupa development 96 h after application (p<0.026, $F_{5,16}$=3.46) with a reduction in egg to pupa survival in 6 and 12% FR (p<0.004, t_{16}=3.34 and p<0.033, t_{16}=2.24 respectively) (Figure 34b). Egg to adult survival was also reduced 168 h after application (p<0.038, $F_{5,16}$=3.11) (Figure 34c) at 100% FR compared to the control (p<0.057, t_{16}=2.05).

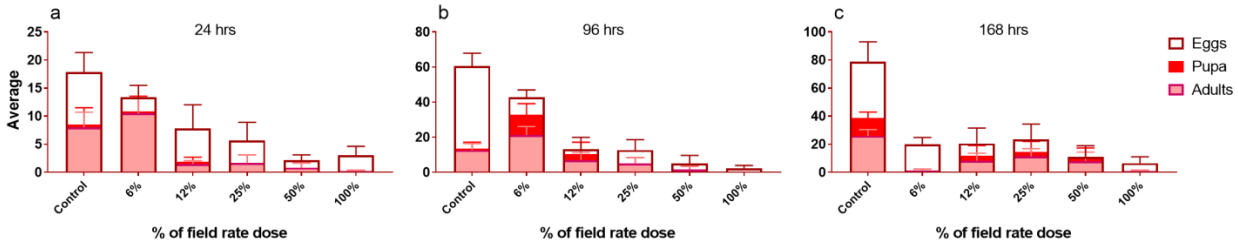


Figure 34. Survival of *D. suzukii* offspring life stages (a) 24, (b) 96 and (c) 168 h after parents treated with spinosad dilutions of field rate (150ml/ha).

Chrono-toxicity

Both mortality and the total number of *D. suzukii* eggs laid in each pesticide and time combination (04:30 and 12:00) was reduced in comparison to the control (Figure 35). There was a significant difference between the control and LC50 for the number of eggs laid per live female treated with cyantraniliprole, lambda-cyhalothrin and pyrethrum, however, no difference was observed in the number of eggs laid per female between the control and *D. suzukii* treated with the LC50 dose of spinosad.

No significant difference was identified in mortality, total eggs or eggs per female between LC50 dose treated *D. suzukii* in relation to time for any of the pesticides. Hence, in this study no chrono-toxicological effects were observed.

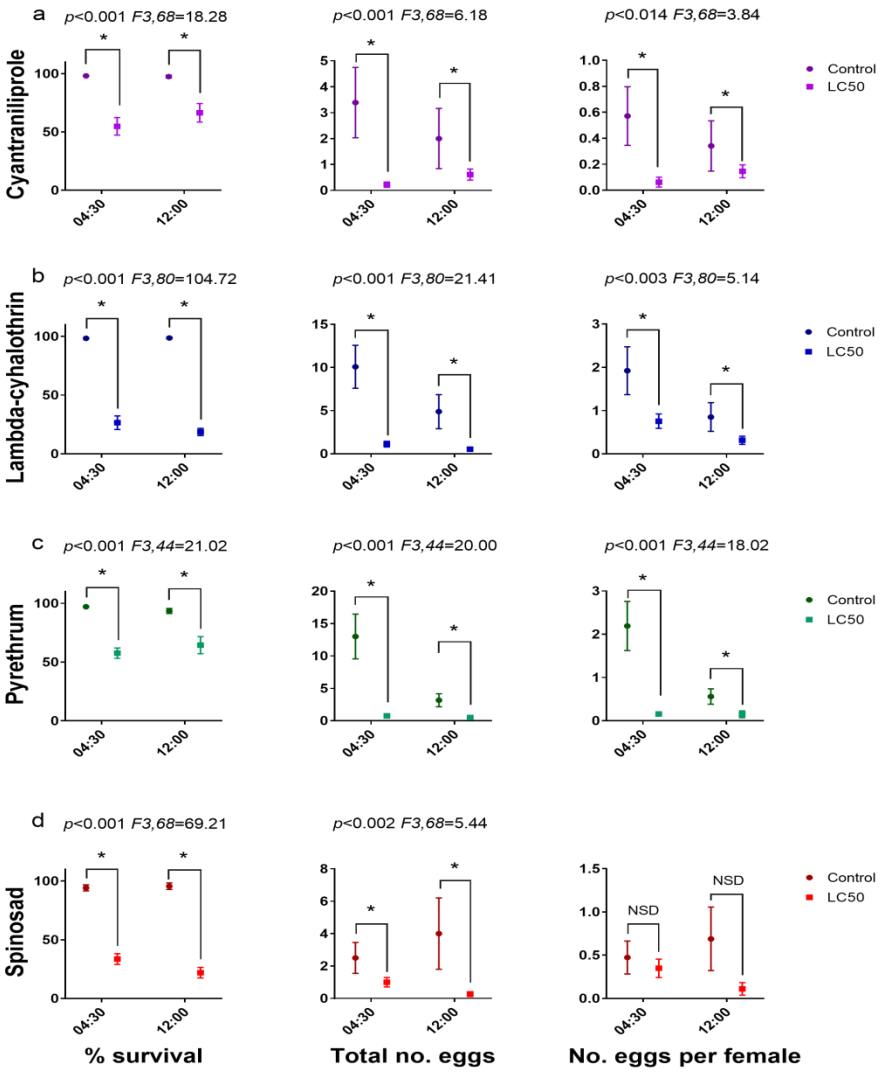


Figure 35. The effects of chrono-toxicity on; *D. suzukii* adult mortality, total number of eggs and number of eggs per female treated with the LC50 of (a) cyantraniliprole, (b) lambda-cyhalothrin, (c) pyrethrum and (d) spinosad.

Discussion

Experiment 1: Investigating the locomotion of D. suzukii under different social conditions and temperature and light cycles

Within the data presented here we have displayed the wide variation in locomotion behaviour produced by slight changes in methodology. Although many of the conditions used are not novel, the influence of such a wide range of factors, including social housing, has not been investigated in such depth to date in *Drosophila suzukii*. Observing individual, unmated flies in constant conditions has provided invaluable knowledge surrounding the circadian clock (Chang, 2006). However, to gain a better understanding of wild like behavioural patterns, more natural parameters are needed. It has already been identified that the mating statutes of *D. suzukii* influences patterns in locomotion behaviour in *D. suzukii* (Ferguson et al., 2015). As individuals in the field would not stay virgin for long (Revadi et al., 2015) and as it is mated females that are the root of this pests' negative impact, in the work presented here all observations were made on mated flies in both individual and social housing groups.

From the range of social situations evaluated across all environmental conditions, sex composition, space and setting all influence locomotion behaviour. This can be seen in the locomotion profiles. When collected in semi-field natural conditions and under constant darkness in the laboratory, all social housings displayed very similar patterns in profile. In both laboratory and field collected profiles most activity occurred during the 'day' and very little occurred during the 'night' even when lighting cues were removed. In the semi-field and their corresponding laboratory mimic collections the female groups and the mix sex groups are most visually similar in activity profile. Meaning that if appropriate environmental conditions are used it is possible to gain an understanding of locomotion behaviours of flies exposed to natural conditions from laboratory-based assays. Within our investigation the variation between individual flies and group housed flies, monitored under the same environmental conditions, emphasise the impact of social interaction on locomotion behaviour.

The importance of observing behaviours under social conditions has been stated by several groups who highlight that interaction between individuals will affect behaviour not only in species that have complex social structure, such as the honey bee, but also in less complex systems, including *Drosophila* species (Bloch et al., 2013, Fujii et al., 2007). Mating and

courtship in *D. suzukii* involves mobile rituals of wing flashing and circling and would surely increase activity levels in populations of flies (Revadi et al., 2015). De at al., (2013) modified lighting factors 'under otherwise semi-natural' conditions and monitored locomotion behaviour of *Drosophila melanogaster,* to determine how activity was affected by changes in light. They also hypothesised that there would be changes in activity due to courtship and concluded that the morning peak of activity in *D. melanogaster* is due to 'courtship-related locomotion'. Within our assay, individual flies housed in the narrow cuvettes frequently displayed a different locomotion profile than groups in the large population vials. Although, unlike De et al,. (2013), we do not see variation in morning activity peaks between individual and group housed flies under the semi-field or any of the laboratory collected locomotion profiles in *D. suzukii*.

The activity profiles of mix sex groups and female group's collected under the laboratory mimic cycles, were most representative of those collected under natural conditions in the semi-field collections. Groups of males resemble individual males in general activity pattern and both varied from mix sex groups. Individual males and females were observed in large population vials with in the laboratory to see how the increase in space affected activity patterns. Within these profiles, locomotion rhythms were visually very different from not only groups within population vials but also individuals in narrow cuvettes highlighting the variability in activity pattern produced by an increase in space alone.

Within both laboratory and semi-field conditions, the common anomalous social settings were individual males, individual males in population monitors, individual females in population monitors and groups of males indicating that these housings are more sensitive to changes in artificial lighting events. The use of these as an indicator of overall population activity may lead to a misunderstanding of increased activity. Mix sex groups and female groups were typically found within the centre of each environmental cluster again suggesting that groups that contain females could be used as a reliable indicator of what an average population's locomotion behaviour is under a condition.

In the *D. melanogaster* circadian clock, social interaction impact on activity has already been identified as a 'nonphotic' cue, capable resetting circadian rhythms (Levine et al., 2002). In the work we present here we also see an effect of social housing on maintaining circadian rhythms in *D. suzukii*. Under the constant conditions, DD 23ºC, groups of flies retained rhythmicity for a longer time than individual flies in the smaller cuvettes as derived from the actograms. As the actogram displays an average activity from a sample group, it could be conceded that the higher number of individuals in the mix sex groups results in a dampening of arrhythmic behaviour. However, group males contained the same number of flies as the individual males in the analysis, and still we see a loss of rhythmicity within the

individuals. Rhythmicity remained within group females and mix sex groups longer than group males and displayed day and night peaks and troughs in locomotion activity throughout the assessment period, even 7 days without environmental cues to regulate rhythms. Groups of flies also showed a much stronger rhythmicity than individual flies with all three defined as rhythmic by their RRP value. Group females had the strongest rhythm followed by the mix sex group and group males respectively while individual males and females fell into the weakly rhythmic category. Typically an organism's clock runs on a roughly 24-hour period length, synchronised with one turn of the earth or one day-length (Bollinger and Schibler, 2014) when exposed to environmental cues. Group housed flies held under consent conditions within our assays displayed period lengths closer to 24 hours in comparison to individual males and individual females, which showed an increased period of 27.7 and 25.8 hours. The combination of identifying RRP and period length enables us to identify that groups of flies are entraining to socially conserved rhythms.

Within each environmental condition there were significant differences in the average activity counts per hour per fly dependent on both social housing and environmental condition. When evaluating social housing alone, typically individual males had significantly lower average counts per hour per fly than individual females and the various groups housed in population monitors. It had been suggested having a higher density of flies in mixed sex groups (20 individuals) would result in disturbances and therefore higher activity counts than in the single sex groups (10 individuals) or the individual flies. This hypothesis was investigated in an extensive examination of social interactions and its effect on sleep in *D. melanogaster,* conducted by Liu et al (2015). Patterns of individual mated flies were compared with single sex groups of various densities and variations in sleep habits were detected. They found that total sleep decreased drastically as population density increased. However, in our assay, groups of females, which contained 10 individuals, displayed the highest activity counts per hour per fly in 6 out of the 16 conditions. This was higher than any other social grouping including the mix sex groups which contained 20 individuals which, if responded the same as the Liu et al, (2015) study, we would have expected to have the highest activity counts. The increased activity per fly per hour in group females may be due to females searching for additional egg laying sites as *D. suzukii* prefer less dense sites compared to *D. melanogaster* (Mitsui et al., 2006), and to also reduce oviposition competition (Shaw et al., 2018).

Within this research, we have investigated locomotion under both semi-field and laboratory settings. We have not been the only group to observe behaviour under semi-field conditions, however, no other group has, in such large quantities, attempted to promote wild-like behavioural patterns within an artificial environment in *D. suzukii*. Ideally,

observations should be made under semi-field conditions (Kannan et al., 2012) to predict wild-like behaviour, however, there are limitations and restrictions to working in natural conditions. The reproducible control environment provided by the laboratory enables researchers to replicate parameters and provides second chances, which would not occur in the field as no two days are the same. Also, being able to manipulate conditions enables investigations at any time opposed to waiting for environmental conditions to occur naturally in the field. Where semi-field-based assays are not appropriate, controlled environment chambers with gradual changes in light and temperature are optimum when investigating physiological behaviours such as diapause (Nagy et al., 2018). Within these assays we also did not attempt to fluctuate humidity within the laboratory, however, it would cycle within semi-field environments. As humidity is a vital factor in *D. suzukii* development and survival (Hamby et al., 2016, Tochen et al., 2016) environment chambers were kept at a constant 65% to keep mortally low through our observation period. However, here we have displayed that even with a 3 stepping light cycle and without fluctuating humidity we are able to closely reproduce 'wild-like' behaviour in the laboratory when other influencing factors are used.

By producing a standardised methodology to investigate behavioural patterns in *D. suzukii* we can enable the comparison of results from wider groups. As many research groups have varying ways of investigating behaviour, by understanding how factors such as environmental, social grouping and space influence patterns we can discern whether contrasting results are due to methodology or unforeseen reasons i.e. an increase in startle response in individual flies when we know that in groups of flies this would not occur is such an exaggerated way.

Experiment 2: Can we predict oviposition rhythms in the field in laboratory based experiments?

In this study, oviposition rhythms were identified in wild *D. suzukii* populations and then reproduced using simulated light and temperature profiles in the laboratory. Furthermore, we show that laboratory-based experimental set-ups could be a valuable tool for predicting field behaviour across a wide range of environmental conditions. We found a strong relationship between the numbers of *D. suzukii* eggs and the number of adult's emerging following incubation under laboratory conditions. On average 82% of eggs survived to adult emergence, justifying our approach of measuring daily patterns in reproductive success.

Where reproductive success was compared between the light and the dark phase significantly more oviposition occurred in the light. In a laboratory trial lacking temperature cues altogether (12:12 LD at constant 23ºC) egg laying in the light was 2.5-fold higher than in the dark. This difference was strongly enhanced (15.4 fold) when a basic temperature cycle (17 ºC during the light phase/11ºC during the dark phase) was introduced along with a 17:7 LD cycle, all at constant relative humidity. Moreover, wild populations in the field exposed to natural fluctuations in light and temperature showed strong and significant day-time preferences for reproductive oviposition in August (9.1 fold) and October (25 fold), while the opposite was true for the corresponding laboratory assays. In both August and October field trials, there was no consistent peak in reproductive oviposition in relation to sunrise/sunset, indicating that egg laying patterns are not solely reliant on lighting cues to entrain this behaviour.

Lower oviposition occurred during the field trial in October versus the one in August. We attribute this apparent inconsistency to the relative population densities in wild habitats at these times of the year. Overall, 571 eggs were laid under the June conditions in the laboratory in comparison to the 49 and 146 laid for the August and October laboratory trials, respectively. Both October and August laboratory conditions included lower or upper development thresholds temperatures for *D. suzukii* (Enriquez and Coli net, 2017) which is likely to have reduced oviposition rates within these experiments. In the October field trial, peaks in reproductive oviposition occurred from oviposition windows that either encompassed peak temperature or 0-2 hours after. This was also found in the June and October laboratory trials.

Within both the August field and supporting laboratory trial, reductions in reproductive success occurred during time intervals with >30ºC temperatures. Peak reproductive oviposition was delayed until the onset of relatively cooler temperatures during dusk. Moreover, day/night differences in field reproductive oviposition were lower during the

August than the October trial. These observations suggest that the daily profile of *D. suzukii* oviposition in the field is expected to reach its peak with the daily temperature maximum in the early afternoon. However, when temperatures exceed the preferred range for oviposition, the daily oviposition profile may become bimodal and exhibit daily maxima just before dusk. This is indicated by the October field data, the supporting laboratory trial and the June laboratory trial in which the extreme temperatures are not experienced. This hypothesis is further corroborated by the results of Evans et al. (2017), who found that in a day vs. night assay conducted in the context of high day-time temperatures (30-37°C), significantly more eggs were laid during the dusk-night-dawn interval than during core daylight hours. Temperatures of ≥30°C exceed the high temperature survival limit of *D. suzukii* (Tochen et al., 2014) and may trigger escape behaviour to cooler locations if available. Indeed, Van Timmeren et al. (2017c) observed a decrease in the number of wild *D. suzukii* on blueberry bushes during the warmest time of the day, when temperatures averaged 30°C.

Our ability to reproduce daily patterns of oviposition in the field by recapitulating photoperiod and daily temperature gradient in the laboratory is illustrated for the August condition, where oviposition counts exhibited no significant differences between laboratory and field assays. This was true both for pairwise comparisons per time interval and for temperature interval. Due to the sparse data under October field conditions no events were registered for some daily time intervals and these then emerged as times of day with significantly lower reproductive success rates in field versus laboratory. Significant differences between October laboratory and field conditions were also noted between reproductive success at two intermediate temperature intervals. Nevertheless, the peaks in reproductive success during both field and laboratory October conditions coincided with the afternoon intervals and the temperature maxima. Thus, the laboratory trials for both August and October conditions successfully identified the daily peak in reproductive oviposition observed in the associated field trials.

Locomotion rhythms are far easier to collect and a more widely used parameter in circadian rhythm investigation in comparison with oviposition rhythms. Since both oviposition (Evans et al., 2017, Lin et al., 2014) and locomotor activity (Hamby et al., 2013, Ferguson et al., 2015) exhibit daily rhythmicity in *D. suzukii*, we explored the association between these two periodic behaviours under in identical environmental cycles in the laboratory. Although the relative rates of locomotor and oviposition activity seemed to be generally matched well in the mid-afternoon to dusk interval, locomotor activity also spiked at other times. Increases in locomotor activity following lights-on or lights –off are known as startle responses and are not uncommon in *Drosophila* locomotor activity (as discussed in Experiment 1) The

locomotor activity peak observed around mid-day in the August experiment may represent an escape response triggered by noxious heat. Consistent with this idea, this locomotor activity peak precisely coincides with the >30°C temperature peak. As *Drosophila* are ectotherms they do not maintain a constant body temperature and use the environment to regulate it, i.e. by moving into the sunlight when cold and moving to shade when hot (Abram et al., 2016). The increase seen in the mix sex group locomotion activity as temperatures reached 30°C could be the result of the flies increasing movement to try and locate a cooler niche. In the field, flies can escape from the rising temperatures and locate cooler areas to wait out the high heat. The resulting effect may be seen in both the laboratory and field oviposition trials as a reduction in oviposition as females are focusing on their own survival rather than egg laying. As eggs are immobile and unable to escape high temperatures, the reduction in reproductive oviposition in August could also be due to the females not depositing eggs in unfavourable environmental conditions and retaining eggs until conditions become optimum (Dillon et al., 2009, Horváth and Kalinka, 2018). Such retention of eggs by *D. suzukii* females until more favourable conditions occur has been termed 'brood care' (Zerulla et al., 2017).

Experiment 3: How will the presence of another species affect oviposition behaviour in D. suzukii?

The aim of this study was to investigate whether the presence of an opposing species in oviposition substrates would disrupt offspring emergence. In the next-generation emergence experiments, *D. melanogaster* emergence was not affected by *D. suzukii* as there was no difference in emergence from pre-inoculated and blank media. When given a choice, female *D. melanogaster* oviposited in pre-inoculated medium even when offered a resource free from *D. suzukii*. As with many native *Drosophila* species, *D. melanogaster* utilise damaged and decomposing fruit for egg laying and may not perceive the presence of another species as detrimental.

D. suzukii next-generation emergence was significantly lower from substrates pre-inoculated with *D. melanogaster* compared with a blank medium. It is suggested that this could be attributable to cannibalistic tendencies of some *Drosophila* species which occur when nutrition is restricted (Ahmad et al., 2015). Morphological defects can be a visual indication that cultures have been maintained on diets lacking nutrition (Vijendravarma et al., 2010). Although no quantitative measurements were taken, there was no noticeable reduction in body and wing size in our competition experiment to indicate diet restriction which promotes cannibalism, or, in this case, interspecific predation.

In our third treatment in the next-generation emergence experiment, *D. suzukii* females were given a choice of blank medium or medium pre-inoculated with the eggs and larvae of conspecifics. There was no significant difference in emergence from these two medium options. If significantly more *D. suzukii* emerged from the pre-inoculated medium, this could have indicated oviposition aggregation pheromones as found in some *Drosophila* species (Symonds and Wertheim, 2005, Wertheim, 2001). However, in wild populations, if given a choice, *D. suzukii* eggs are typically deposited either singly into fruit or in very small clutches (Mitsui et al., 2006), indicating a *D. suzukii* preference for oviposition sites low in egg counts or free from both *D. melanogaster* and conspecifics.

To identify the point at which competition occurred, a female *D. suzukii* oviposition choice experiment was conducted. In this assay, we found, on average, 23 *D. suzukii* eggs on blank medium compared with an average of 3 *D. suzukii* eggs on medium that had been pre-inoculated with *D. melanogaster*. This finding does not eliminate the possibility of larval competition but does indicate a reluctance of female *D. suzukii* to lay eggs where there are eggs of *D. melanogaster* initially. It does not remove the possibility that the reduction may be a result of the *D. melanogaster* larvae predating on *D. suzukii* eggs. However, cannibalism or interspecific predation primarily occurs in either low-nutrient medium or

highly populated substrates on older immobile larvae. Vijendravarma et al. (2010) found that, to promote cannibalism, larval density had to exceed 15 larvae per gram of standard medium. In our experiments, an average of 10 g of standard medium was used, meaning that, to promote cannibalism, each dish would need larval densities of 150 larvae. The highest possible density in the pre-inoculated medium was 3.4 larvae per gram, 4.4 times lower than that to promote predation.

From our results, we can conclude that a reduction in *D. suzukii* emergence from *D. melanogaster* pre-inoculated medium was at least partly attributable to female oviposition choice. In natural conditions, the niche that *D. suzukii* occupies means that they do not normally need to compete with other *Drosophila* species for egg-laying resources until ripening fruits become scarce. However, in assays performed by Bernardi et al. (2016) when given a choice of varying fruit ripeness, and with no competition, significantly more *D. suzukii* eggs were laid on, and adults emerged from, ripe and overripe fruit compared with ripening fruit; a trend also found by Lee et al. (Lee et al., 2011, Lee et al., 2015) in comprehensive investigations. There was also no significant attraction preference for under ripe fruit volatiles in comparison to ripe or overripe volatiles in approach assays (Keesey et al., 2015). Keesey et al. (2015) discuss the point that ripening fruit volatiles alone do not explain *D. suzukii*'s preferences for ripening fruit as an oviposition site.

Cis-vaccenyl acetate (cVA) is a complex, multifunctional, male-produced pheromone used in courtship, aggression and aggregation signalling inmost *Drosophila* species (Datta et al., 2008). However, cVA appears to have a disruptive effect on *D. suzukii*, and, when applied to males, resulted in reduced mating (Dekker et al., 2015). This change in pheromone perception in *D. suzukii* could be the reason females oviposit into under ripe fruit when other species have utilised overripe fruit, detectable by the presence of cVA or a similar olfactory cue. If so, it is possible that the volatiles released by pre-inoculated resources could act as a natural repellent to female *D. suzukii* searching for oviposition sites. This would suggest that *D. suzukii* utilise under ripe fruit for oviposition to reduce competition with other *Drosophila* even though, when there is no competition, riper fruits would be preferable. Olfactory repellents have been used for many years to deter biting insects such as mosquitoes and have been successful in reducing oviposition in laboratory assays in some crop pest insects including cabbage moth, *Mamestra brassicae* (Seljåsen and Meadow, 2006), and sweet potato whitefly, *Bemisia tabaci* Gennadius (Bleeker et al., 2011). Although there is a range of possible oviposition repellents for *D. suzukii* control, none are based on volatiles of pre-inoculation. If *D. suzukii* females avoid laying eggs in fruit that has been previously infested, then it may be possible to synthetically produce compounds or other signals characteristic of infested fruit and use them as egg-laying repellents.

Experiment 4: Is there any impact of the circadian clock on plant protection product susceptibility?

Within this study, we were unable to detect any impact of chrono-toxicity on mortality or oviposition rates of *D. suzukii* to any of the plant protection products applied between the two time points. However, we did identify previously unobserved interactions with sub-lethal doses of insecticide on adult mortality, oviposition and the survival of immature stages. As chemical applications play a large role in controlling *D. suzukii* (Andreazza et al., 2018, Beers et al., 2011, Bruck et al., 2011, Cowles et al., 2015, Pavlova et al., 2017), increasing the knowledge on how it interacts with plant protection products is of vital importance. Both positive and negative impacts of sub-lethal doses were observed within this investigation, which has subsequent consequences for both *D. suzukii* population growth and insect resistance management of this pest.

The application of lethal doses of plant protection products to crops is a legal requirement (IRAC), however, it is not uncommon for pests to come into contact with sub-lethal doses (Coats, 1991) as plant protection products degrade after application due to environmental factors including light, temperature, rainfall and humidity (Fenner et al., 2013). The impact sub-lethal doses had on mortality varied greatly between the plant protection products applied. For example, flies treated with 6 % FR of cyantraniliprole (67.5 ml/ha), 6 % FR lambda-cyhalothrin (4.5ml/ha) and 6 % FR pyrethrum (144 ml/ha) had significantly higher mortality in comparison to the control. However, no difference in mortality was identified in flies treated with 6% spinosad FR (9ml/ha) in comparison to the control.

There was no difference in mortality over time for flies treated with any dose of lambda-cyhalothrin, pyrethrum or spinosad. Hence, if they survived the first 24 h after exposure they survived for at least 168 hrs. There was, however, a reduction in survival from 24 – 168 h after application with 3% FR cyantraniliprole (33.7 ml/ha). This displays that there is a gradual toxic effect on flies treated with this dose. This delay in mortality has also been observed in honeybees, in which the impacts of sub-lethal doses of imidacloprid (class; neonicotinoid) are not identified until 30 days after treatment application (Rondeau et al., 2014).

In most cases there was a reduction in total eggs laid when adults were exposed to the range of doses of each plant protection product. This reduction was the result of decreased mortality in adults. Consequently, the number of eggs per live female was calculated to distinguish if there was any impact of dose on reproductive output of surviving females. By

calculating the numbers of eggs laid per live female, we are able to identify whether females that survived the initial spray event were as reproductively active as those not treated. For cyantraniliprole, significantly fewer eggs were laid per female when treated with 3-50% FR (33.7-562.5ml/ha) 24 h after application. However, at 48 h there was no difference between 3% (33.7 ml/ha) treated *D. suzukii* and the control, and at 96 h there was no difference between 0.75-50% FR (8.4-562.5ml/ha) treated *D. suzukii* and the control. This trend could be interpreted as the flies recovering from the spray treatment as time passes. By the end of the assessment period, all surviving females treated with all doses were laying the same number of eggs as the untreated control. Hence, if adult *D. suzukii* survive an initial spray of cyantraniliprole there were no lasting negative effects on oviposition. This trend was also overserved in *D. suzukii* exposed to lambda-cyhalothrin and pyrethrum, with a reduction in total egg laying overall but a recovery of number of eggs laid per female from 48 h onwards in all doses. Spinosad treated *D. suzukii* were the exception to this trend, with a reduction in the number of eggs laid per female occurring in 6% FR (9ml/ha) from 96-168 h after exposure. However, this was not the case in *D. suzukii* treated with 12-100% FR (18-150ml/ha) spinosad, in which, by 168 h females were laying as many eggs per female as those in the untreated control.

Within our research, more eggs were laid in total and a higher number of eggs per female were laid by flies treated with 0.33% FR (3.7ml/ha) cyantraniliprole; the only incidence this occurred within this investigation. This indicates that the application of the lower dose, promoted an increase of egg production. This has also been seen in other insects, in which a positive response occurs after an application of pesticide (Calabrese and Mattson, 2011). The beneficial impact of sub-lethal doses on an organism, known as hormesis (Müller, 2018), has been identified as having a positive effect on reproductive success a range of pest species (Cutler, 2013) including improving sex pheromone responses in some *Lepidopteran* species leading to increasing mating success (Lalouette et al., 2016, Rabhi et al., 2014) and increasing reproductive rate in aphid species (Ayyanath et al., 2013, Wang et al., 2017, Cutler et al., 2009). Hence the exposure of *D. suzukii* to sub-lethal doses, either directly or indirectly via degradation of pesticides over time (Fenner et al., 2013), could increase fecundity with exposure to some plant protection products.

In this study, the majority of sub-lethal and lethal doses did not reduce *D. suzukii* survival or decrease the numbers of eggs laid per female. However, not all eggs laid within this assay developed through to maturity, and the impact of treating parent flies with lowered insecticide doses, had a negative effect on offspring survival. For both lambda-cyhalothrin and spinosad, there were reductions in egg survival at some doses resulting in a low number of offspring reaching maturity. These ‘transgenerational’ effects have been

observed in a number of species and typically include higher mortality of offspring (Costa et al., 2014, Szabó and Bakonyi, 2017). In our assay, possibly the most concerning result we identified was that there was no difference in the number of eggs an individual female laid 48 h after spray application with 100% FR spinosad (150ml/ha) compared to the control. However, there was a reduction in the survival of eggs through to pupal development and eggs through to adult emergence at 96 and 168 h after spray respectively. To summarise; a low number of females treated with the field rate of spinosad survived, and egg laying recovered to the level of the control, but of the eggs being deposited, only very small number survived through to adult emergence.

We did not see a reduction in survival of egg to adult offspring development in flies treated with cyantraniliprole or pyrethrum. As there was no reduction in eggs reaching maturity, an overall population increase occurred in flies treated with 0.33% FR (3.7ml/ha) cyantraniliprole. Although we only observed the first generation, transgenerational hormesis has been identified within aphid species, increasing population growth up to 4 generations after a treatment application (Ayyanath et al., 2013). When subsequent generations of an insect are repeatedly exposed to sub lethal doses of plant protection products, there is an opportunity for resistance to those chemicals to occur (Guedes, 2016). The combination of these factors could result in not only larger populations of a pest but also an increase in the possibility of insecticide resistance (Guedes et al., 2017, Brevik et al., 2018). This is a risk with *D. suzukii*, due to its relatively short development time, overlapping generations (Emiljanowicz et al., 2014, Grassi et al., 2017, Hamby et al., 2016, Revadi et al., 2015, Tochen et al., 2014) and frequent exposure to plant protection products (Wiman et al., 2016).

Conclusion

The main aim of this PhD is to investigate daily and seasonal rhythms of *Drosophila suzukii,* which will lead on to formulate recommendations for UK growers to aid combating this pest. In order to do so, understanding what is influencing the patterns of behaviour is key. By identifying the determinants of daily rhythms of *D. suzukii* activity this may help predict field behaviour.

The work documented here greatly increases the knowledge surrounding *D. suzukii* behaviours and what influences them; an area of research that has been highlighted as important by several research groups (Ferguson et al., 2015, Silva-Soares et al., 2017, Hamby et al., 2016, Hamby et al., 2013, Harris et al., 2014). Within this thesis a range of both biotic and abiotic factors that can regulate, influence and disrupt *D. suzukii* behavioural rhythms has been explored. In Experiment 1 parameters that influence locomotion activity are evaluated. The primary aim was to identify the determinants of daily rhythms of *D. suzukii* activity including the impact of the circadian clock, light, temperature and social housing on locomotion behaviour. As locomotion behaviour is one of the easiest behavioural outputs to observe, we believed that by identifying the parameters needed to produce wildtype activity behaviour, we would then be able to apply these conditions to observe other key behaviours in this species.

We were able to identify variations in locomotion activity caused by simple alterations to methodology, even under standard environmental conditions including a basic light cycle and constant temperature. There were great differences in general activity levels and the behavioural patterns between different social groupings. We found that groups of flies that contained females (i.e. groups of females only and mixed sex groups) had the least variation between profiles collected under natural conditions and those collected under the recreated conditions within the laboratory. Individual males, which are typically the focus for circadian rhythm investigations, gave consistently anomalous results within each environmental condition and were not a good representation of overall *D. suzukii* activity patterns. This suggests individual males are not an appropriate sample group when investigating behaviour rhythms of this species. Within the laboratory, individual males, groups of males and individual males or females within population monitors, were more sensitive to lighting events and displayed hyperactivity at dusk and dawn lighting changes. This was not seen in groups of females or mixed sex groups. Groups of females, mixed sex groups and groups of males remained rhythmic for longer in constant darkness at a constant temperature than both males and females housed as individuals. This displays an

element of social entrainment in conditions void of environmental cues which has been seen in other *Drosophila* species but had not been documented in *D. suzukii*.

By altering temperature and light cycles we have been able to identify the impact these factors have on locomotion activity. We have used fluctuating and constant environmental conditions, and by comparing a wide range of environmental conditions have identified those that would be most appropriate in making behavioural predictions within a laboratory. We have demonstrated clear separable impacts of the circadian clock, light, temperature, and social housing on *D. suzukii* behavioural rhythms. Semi-field conditions were deemed the optimum environmental condition to investigate wild-like behavioural rhythms. However, as the field simulated conditions within the laboratory had the fewest significant differences in normalised activity counts compared to the semi-field, it seems that a realistic temperature and light cycle can promote wild-like behaviour in laboratory conditions using groups of females.

With the greater understanding of how light and temperature within a laboratory can influence activity in this species, we progressed within Experiment 2 to investigate oviposition patterns in *D. suzukii*. As with Experiment 3, we aimed to recreate wildtype behavioural patterns within an artificial environment. Field trials were deployed to record oviposition rhythms of wild and unrestricted *D. suzukii* populations. For the 2 time points in the year that this field trial was done, August and October, we found that oviposition primarily occurred during daylight hours and extreme temperatures experienced during the August field trial resulted in disrupted oviposition. When temperatures exceeded 30ºC oviposition rates declined and when temperatures dropped to more optimal conditions, oviposition was reinstated. In the October field trial oviposition peaks coincided with peak temperature typically around midday. Oviposition rhythms under natural conditions had previously been investigated by Evans et al. (2017) who found contradictory results to ours. They found more oviposition occurred during the night, however a review of their results showed there was no survival of females during the day in which temperatures exceeded 37ºC and 100% mortality occurred. This contradiction confirms that behaviour assays should be conducted under temperature and light cycles appropriate to country of interest, as these factors vary greatly between location and are the driving factors in regulating the circadian clock and, therefore, behavioural rhythms.

The environmental conditions experienced during the field trials were recreated in a laboratory to identify whether laboratory strains of *D. suzukii* would produce similar oviposition patterns under the recreated environmental conditions. Within the laboratory trials, oviposition rhythms were a good visual and statistical representation of oviposition patterns collected within the field trials. In the August laboratory conditions, oviposition also

fell at temperatures above 30ºC indicating this behavioural response is conserved in both wild and laboratory strains of unrestricted *D. suzukii*. The fact that natural rhythms of oviposition and locomotion can be readily induced within an artificial laboratory setting means that if the correct environmental and social settings are used predictions can be made for spesific times of interest. In additional experiments within the laboratory we were able to explore the impact that light and temperature had on oviposition. Under a basic 12:12 L:D cycle at a constant 23 ºC, significantly more eggs were laid during the light phase than in the dark. When a stepping cycle (i.e. one constant temperature during the day and a different constant temperature during the night) was combined with a longer day length (17:7 LD 17/11°C) a stronger preference for oviposition during the light was produced. The final stage was to introduce a fluctuating temperature and light cycle which further increased the strength of daylight oviposition, stressing the point that more realistic parameters are needed within laboratory assays on behaviour.

The results from the first two experiments indicate that predictions of oviposition and locomotion can be made within an artificial laboratory setting when the correct environmental and social groupings are used. This is an extremely useful tool and can be used if environmental conditions of key times in the commercial year are known. In future, behavioural assays on *D. suzukii* should adhere to these parameters which can then by applied to other biological traits such as feeding, courtship and mating.

Within the first two experiments we have been able to investigate how environmental conditions can influence and disrupt behavioural patterns. In Experiment 4, we have continued to investigate how to disrupt behavioural patterns in this species by introducing a competitor to an egg laying site. In the laboratory, we have been able to explore how the presence of *D. melanogaster* can significantly reduce the number of *D. suzukii* offspring emerging. It appears that the reduction in *D. suzukii* offspring emergence is due to fewer eggs being laid, initially, in the presence of a competing species rather than predation of larvae. In choice experiments, more eggs were laid, and more offspring emerged from blank substrates free from eggs and larvae of *D. melanogaster* than in substrates that contained *D. melanogaster* eggs and larvae. In the reciprocal experiment, there was no impact of *D. suzukii* presence on *D. melanogaster* egg laying and offspring emergence. Further investigation into the exact point of competition is needed to understand the interactions between these two species, however it is highly likely that a volatile signalling compound causes this disruption.

As the presence of another species deters oviposition by *D. suzukii* it is possible to combine this information with our understanding of oviposition behavioural rhythms in the field. A combination of these two factors could lead to the development of an oviposition deterrent

which is specifically applied or released during peak oviposition time and incorporated in to an IPM program to target *D. suzukii*.

In the final experiment we take our understanding of the circadian clock, and how it is influenced by environmental and social conditions, and explore the effects of time on insecticide susceptibility. This 'chrono-toxicity' has already been identified in *D. suzukii* to malathion, however this is not a EU approved product. An organism's sensitivity to toxins varies throughout the day due to the cycling of detoxification genes, which are under control of the circadian clock. Therefore, there may be times of the day that *D. suzukii* is more sensitive to plant protection products than at other times. Populations of *D. suzukii* were exposed to a realistic temperature and light cycle typically found within early summer in the South East of England in the laboratory. Within this experiment, four commonly used, UK approved, plant protection products were applied to groups of *D. suzukii* at two time points within the day. Plant protection products were applied at the peak and trough of the temperature cycle, which coincided with peak and trough *D. suzukii* activity levels. These time points were selected to ensure that the method was accessible to all growers as simply understanding temperature cycle within a crop is sufficient to use this method. Also this design allows us to investigate the theory that a higher level of activity would increase mortality due to increased contact via increased movement. Within these experiments we did not detect any changes in insecticide susceptibility between the two time points in terms of mortality or oviposition rates. However, we were able to gather valuable information on the impact of sub-lethal doses on *D. suzukii* oviposition and transgenerational effects. There was a range of responses that varied between positive and negative impacts seen across the four plant protection products tested. Hormesis was found to occur in flies treated with the lowest dose of cyantraniliprole with an increase in oviposition rates and more eggs laid per female. There were no negative transgenerational effects at this dose and there was no significant reduction in offspring survival. In groups treated with lambda-cyhalothrin and pyrethrum there was a significant impact on mortality between the control and lower doses of insecticide, however there was no significant reduction in the number of eggs laid per live female by the final assessment. In flies treated with the highest doses of spinosad, including field rate, there was a significant difference in mortality but no significant difference in the number of eggs laid per live female by the final assessment. However, there were transgenerational effects and not all oviposited eggs survived through to adult emergence.

The work carried out within this thesis has identified the correct parameters needed to make predictions of behavioural rhythms of *D. suzukii* within an artificial environment. We have been able to validate these conditions in field experiments on wild populations. We propose standard conditions for future behavioural assays within the laboratory to promote wildtype

behavioural patterns. We have also been able to disrupt oviposition behaviours by introducing a competing species and propose how this work could be investigated further. We have furthered the knowledge surrounding how this species interacts with plant protection products and highlight the effects of hormesis, stressing a need for resistance management in *D. suzukii*. The information gathered should be applied when addressing IPM strategies for dealing with this pest. Having an increased understanding of behavioural rhythms in *D. suzukii* could result in a more strategic and targeted approach to deploying or applying control methods to combat this pest.

Knowledge and Technology Transfer

Poster presentation:

- The UK clock club winter conferences, Edinburgh, December 2015
- The UK clock club summer conference, Coventry, July 2016
- SCI young researchers in crop sciences, Berkshire, United Kingdom, July 2016
- IOBC International Conference on Integrated Fruit Production, Thessaloniki, Greece September 2016
- AHDB The Studentship Conference, Warwickshire, November 2016
- The UK clock club winter conference, Oxford, December 2016
- AHDB Tree fruit day, NIAB EMR March 2017 (winner of poster award)
- The UK clock club summer conference, Bristol, July 2017
- University of Southampton Post-graduate day, July 2017
- NIAB PhD day, Cambridge, November 2017
- Society for Research on Biological Rhythms congress, Florida, May 2018
- European Congress of Entomology, Naples, July 2018

Flash presentation and poster:

- AHDB Tomato Conference, Warwickshire, 29th September 2016
- AHDB The Studentship Conference, Warwickshire 16-17th November 2016

Oral presentation:

- SWD working group meeting, September 2016
- AHDB Soft fruit day November 2016
- AHDB Tree Fruit panel presentation November 2016
- AHDB Tree fruit day March 2017
- International Horticultural Research Conference July 2017
- AHDB The Studentship Conference, Warwickshire, November 2017 (winner of final year presentation award)
- University of Southampton, Environmental biosciences symposium February 2018
- University of Southampton Post-graduate day Conference June 2018

Industry tours

- AHDB studentship industry tour July 2017

Successful grant applications

- GCRI travel grant, September 2016, £500.
- Worshipful company of fruiterers, September 2016, £300
- GCRI travel grant, May 2017, grant returned due to conference not being attended
- GCRI travel grant, July 2018, £600

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