Implications of sub-lethal rates of insecticides and daily time of application on *Drosophila suzukii* lifecycle

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Abstract

The circadian clock is responsible for time keeping within an organism and influences not only behavioural patterns, but also physiological rhythms including toxin susceptibility. *Drosophila suzukii* Matsumura is a global horticultural pest, and identifying rhythms in insecticide susceptibility could contribute to improving integrated pest management practices. To determine whether time of application influences mortality, LC50 rates of cyantraniliprole, lambda-cyhalothrin, pyrethrum and spinosad were applied to groups of adult *D. suzukii* at two different times of day. Insecticides were directly applied using a benchtop sprayer. We found no influence of time on mortality or oviposition for any of the four insecticides applied. However, several discoveries were made regarding the impact of sub-lethal and lethal rates on *D. suzukii* mortality, oviposition and offspring survival over time. In most cases, all surviving females recovered from insecticide rates, and laid the same number of eggs as females treated with the water control. Seven days after application, females that were treated with the field rate of spinosad laid the same number of eggs as the control. The lowest rate of cyantraniliprole resulted in more eggs being laid, initially, with no negative impact on survival of eggs through to adult emergence. However, there were transgenerational impacts of egg to adult survival when parents were treated with sub-lethal rates of spinosad and lambda-cyhalothrin. Although no impact of daily phase of application was detected within this assay, the information surrounding how *D. suzukii* interacts with lethal and sub-lethal rates of insecticides is of great importance, especially for the resistance management of *D. suzukii*.

Keywords: Hormesis; Resistance management; Transgenerational impacts; Rate response; Spinosad; Lambda-cyhalothrin; Cyantraniliprole; Pyrethrum.

1.1 Introduction

Drosophila suzukii Matsumura has been at the forefront of entomological research on a global scale since the late 2000s, as its geographical range rapidly increased (Asplen et al., 2015). *D. suzukii* is one

of only a few species of *Drosophila* able to oviposit within ripening, undamaged soft- and stone-fruits, resulting in economic and yield loss worldwide (Lee et al., 2011, Walsh et al., 2011). Once *D. suzukii* eggs hatch, the larvae feed upon the flesh of the fruit, causing the fruit to collapse making it unmarketable (Emiljanowicz et al., 2014, Revadi et al., 2015, Rota-Stabelli et al., 2013, Silva-Soares et al., 2017). Additional costs of controlling this pest, such as insect-proof mesh, and increased crop hygiene levels and picking frequency, are unavoidable if growers are to ensure producing a commercially profitable crop (Del Fava et al., 2017, Farnsworth et al., 2017, Mazzi et al., 2017).

Insecticides have played a key role in the control of *D. suzukii* since its dispersal from Asia into new regions of the world (Schetelig et al., 2018). This has led to an increase or re-establishment of routine insecticide spray applications which disrupt integrated pest management (IPM) of other pests suppressed by biological control agents (Fountain and Medd, 2015, Roubos et al., 2014). Subsequently there has been a drive to determine the most effective insecticides to reduce population levels and prevent fruit damage by *D. suzukii* (Andreazza et al., 2017, Beers et al., 2011, Cowles et al., 2015, Gautam et al., 2016, Knight et al., 2015, Pavlova et al., 2017, Rosensteel and Sial, 2017, Saeed et al., 2018, Schlesener et al., 2017). Although there are a number of insecticide options to target *D. suzukii,* the efficacy varies. As an example, adult mortality differed between laboratory assays using direct application, from 10% in flies treated with novaluron (group; benzoylureas, inhibitors of chitin biosynthesis) to 100% when treated with zeta-cypermethrin (group; pyrethroid, targets sodium channel modulator), spinosad and spinetoram (group; spinosyn, targets nicotinic acetylcholine receptor) (Bruck et al., 2011, IRAC, 2018). Protection against oviposition ranges from 1 to >14 days, depending on the product used and cropping system (Fountain et al., 2016) and insecticides have also reduced larval and pupal development when applied up to 5 days post-egg laying (Wise et al., 2014). The dependency on chemicals to control this pest is concerning for the future occurrence of insecticide resistance, and this is being investigated in US field populations (Smirle et al., 2017, Van Timmeren et al., 2018). As chemical application has become an integral method in controlling *D. suzukii,* and as

57 regulatory losses of active ingredients become more common (King, 2014) an increased understanding
58 of how insecticides interact with this pest and how their efficacy can be improved is invaluable.

59 Applying insecticides to crops at the peak of *D. suzukii* daily activity could, potentially, increase the
60 possibility of contact between the fly and the chemical. However, previous investigations by Shipp and
61 Otton (1976) identified a more sophisticated mechanism responsible for insecticide tolerance or
62 susceptibility; the circadian clock. The circadian clock is found in most living organisms, from micro-
63 organisms to animals and plants. The circadian clock uses environmental cues, such as light and
64 temperature, to regulate an individual's time-keeping ability (Bollinger and Schibler, 2014, Chang,
65 2006, Chiu et al., 2010) and is a molecular pacemaker responsible for rhythms in behaviour and
66 physiology including xenobiotic detoxification. The latter is the result of the rhythmic cycling of genes,
67 enzymes and proteins associated with the process of detoxification (Allada and Chung, 2010, Paranjpe
68 and Sharma, 2005, Xu et al., 2008). Shipp and Otton (1976) first documented the impact of the
69 circadian clock on insecticide tolerance in *Musca domestica* Linnaeus (Diptera, Muscidae), by applying
70 sub-lethal rates of insecticides at various times of the day. They identified periods of increased
71 susceptibility to DDT (dichlorodiphenyltrichloroethane), dieldrin and malathion at 05:00 under a 14:10
72 light: dark cycle ('dawn' at 06:00). In addition, *M. domestica* transferred to a phase-shifted
73 environmental cycle (in which 'dawn' occurred at 18:00), exhibited a shifted peak in susceptibility (at
74 17:00). This confirmed that susceptibility to the insecticides was influenced by the molecular circadian
75 clock and not the general time of day.

76 In *Drosophila melanogaster* Meigen, several detoxification genes have already been identified as
77 being rhythmic in expression and the relationship between levels and insecticide susceptibility
78 explored (Beaver et al., 2010, Hooven et al., 2009, Misra et al., 2011, Willoughby et al., 2006, Yang et
79 al., 2007). The ability to identify periods of increased insecticide susceptibility in pest insects would
80 have a beneficial impact on pest management. Hamby et al. (2013) identified periods of low and high
81 tolerance of *D. suzukii* to malathion in relation to the expression of genes associated with

detoxification and fly activity levels. Malathion is not approved for use in the UK and Europe and so the exploitation of this result is not an option for these growers. How *D. suzukii* interacts with chemical control could be used to improve best practice techniques, the guidance provided to growers and mitigate insecticide resistance development.

The initial objective of this study was to identify whether the timing of application impacted the efficacy of approved insecticides commonly used on soft fruit in the UK and Europe. However, in the process of fulfilling this aim, further objectives discoveries were made. Therefore, in addition, we explored the impact of sub lethal rates of the tested insecticides on parent fly mortality and the transgenerational impacts on offspring maturation. These responses could have repercussions for population growth and resistance development in *D. suzukii* populations.

1.2 Methods

1.2.1 *Drosophila suzukii* culturing

D. suzukii were maintained at NIAB EMR, Kent, England, from a wild Italian strain collected in 2013 from Trento on the standard BDSC cornmeal diet (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% of 70% ethanol (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/bloomfood.htm)). Populations of *D. suzukii* were housed in glass vials (Kimble Chase 25 x 95 mm Opticlear vials) at 23°C in a 16:8 hour (hr) light: dark cycle at a constant 65% relative humidity within Percival DR-36VL environmental chambers. Cultures were kept genetically diverse by randomly mixing offspring between cultures to reduce inbreeding and were transferred to new food every week.

1.2.2 Fly preparation

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 granulated table sugar in 100 ml distilled water), was placed upon the filter paper. Three to seven-day old *D. suzukii* from mix sex populations were anaesthetised on a CO_2 pad using the Flystuff system (Flystuff (59-121CU) Foot Valve Complete System with Ultimate Fly Pad). Six groups of 6 males plus 6 females were transferred to each Petri dish. The Petri dishes (spray arena) were then 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.

1.2.3 LC50 identification

A Burkard bench top sprayer (Computer Controlled Table top Sprayer, Burkard Scientific Ltd, Uxbridge, UK) was calibrated before each application to ensure the rate of application to the base of the spray arena (i.e. the amount that was applied through the mesh) was within the label rate requirements for each product. This resulted in varying equivalents of water volume per hectare being applied (Table 1). The maximum field rate (FR) for strawberry of pyrethrum (Pyrethrum 5C), lambda cyhalothrin (Hallmark) and spinosad (Tracer) or application rate for cherry of cyantraniliprole (Exirel, which is a formulation not currently approved for use on strawberry) 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 lowest rates. This resulted in the following dilutions of field rate: cyantraniliprole 0.325%, 0.75%, 1.5%, 3%, 6%, 12%, 25% and 50%; lambda-cyhalothrin 6%, 12%, 25%, 50% and 100%; pyrethrum 1.5%, 3%, 6%, 12% and 25%; spinosad 6%, 12%, 25%, 50% and 100%. Dilutions were prepared no more than 30 minutes before direct application by the benchtop sprayer and applied to 6 groups of *D. suzukii* adults at a time (i.e. six groups of 6 males plus 6 females were sprayed per dilution). A control of distilled water was applied for comparison to each insecticide. Applications of rate 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 subjected to the environmental conditions stated in section 1.2.1.

Table 1.Insecticidetreatments, active ingredient and dilution range of field rate (FR) used to spray *Drosophila suzukii.* Target site/mode of action information collected from IRAG Mode of Action Classification Scheme version 9.1 (IRAC, 2018).

Active ingredient (% active ingredient in formulation)	**Target site/mode of Action**	**Trade name and (company)**	**Equivalent of water volume used L/ha**	**Maximum field rate ml/ha**	**Dilution range of % FR**
Cyantraniliprole (10)	Ryanodine receptor modulators	Exirel (DuPont)	580	1125	0.325, 0.75, 1.5, 3, 6, 12, 25, 50
Lambda-cyhalothrin (10)	Sodium channel modulator	Hallmark Zeon® (Syngenta)	400	75	6, 12, 25, 50, 100
Pyrethrum (5)	Sodium channel modulator	Pyrethrum 5C (Agropharm Ltd)	530	2400	1.5, 3, 6, 12, 25
Spinosad (44.03)	Nicotinic acetylcholine receptors	Tracer® (Dow AgroSciences)	580	150	6, 12, 25, 50, 100

Assessments of mortality and survival were made 24 h after spray application and the numbers of eggs laid were counted. At this point, all live flies were transferred to a new ventilated specimen container, with BDSC media, and returned to the environment chamber. Dead flies were discarded. The original specimen container was returned to the environment chamber to allow eggs to develop through to adult emergence. These assessments and transfers were completed at 24, 48, 72, 96 and 168 h 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 life-stage assessed. Assays were repeated twice and results combined for analysis.

1.2.4 Chronotoxicity

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 (11-22^{o}C temperature gradient, 17.5:6.5 light: dark with stepping on/offset of lighting from 0 to 2 and 2 to 4 lighting banks with a 30 minute delay between). Flies were held under these conditions for 3-4 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 (Schlichting and Helfrich-Forster, 2015). 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 section 1.2.2, flies were transferred to the spray arenas. At peak and trough temperature, the 24 hr LC50 for each insecticide was applied using the Burkard sprayer as identified by the Probit analysis from the LC50 assay (see analysis and results). A water control was also applied for comparison. Six groups of 6 males plus 6 females were treated with either the LC50 dose or water control for each time point. After application, flies recovered for 10 minutes within the

161 arena were then immobilised with CO_2 before being transferred to a ventilated 70 ml specimen
162 container containing standard BDSC media. Flies were then maintained within the environment
163 chambers.

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

166 1.3 Statistical analysis

167 Probit analyses were performed using the PROBIT ANALYSIS procedure with a logit link in Genstat
168 (VSN International 2015 Genstat for Windows 18th Edition. VSN International, Hemel Hempstead, UK.
169 Web page: Genstat.co.uk). Probit analyses were performed to identify the LC50 for each insecticide at
170 each assessment point; 24, 48, 72, 96 and 168 hr after application. The model fitted is a logit model
171 with control mortality. The full equation is:

$$\%Mortality = \%CM + (100 - \%CM)\frac{\left(\frac{Dose}{LC50}\right)^{B}}{\left(1 + \left(\frac{Dose}{LC50}\right)^{B}\right)}$$

172

173 Where B is the Slope, %CM is the % Control Mortality and LC50 is the dose for 50% pesticide-related
174 mortality. The LC50 is thus automatically adjusted for the control mortality.

175 The Goodness of Fit of the regressions are also included. As there was over dispersion, the F-test was
176 used. F is calculated as the ratio of the regression mean deviance to the residual mean deviance and
177 tested on (2, residual d.f.).

178 To detect differences in mortality between treatments, a GLM with binomial distribution with a logit
179 link was performed and pairwise comparisons between treatments were tested using difference in
180 deviance analysis for each insecticide individually on each individual day. Male and female mortality
181 was combined to increase statistical power. Significances were quantified using the F-distribution. This
182 was used in preference to the usual approach which uses approximate t-tests based on the differences

between the estimated means with SEDs obtained from the variance-covariance matrix as means near 0 and 100 can give seriously incorrect significances; the “difference in deviance” approach described above avoids this problem. To detect differences in mortality overtime for each rate a GLM with Poisson distribution and a logit link was used.

GLM with binomial distribution and a logit link was used to assess the mortality of total flies for the chronotoxicity data.

All egg counts, for both rate responses and chronotoxicity, were analysed using a GLM with a Poisson distribution and a log 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.

1.4 Results

1.4.1 LC50

The LC50 values were identified for each insecticide for each time point after application (Table 2).

197 Table 2. LC50 and LC90 values of each insecticide treatment, over time, on *D. suzukii*. Values are presented as a percent of recommended field rate (%).

Pesticide	Time	Total number (all doses)	Slope (B) (+/- SE)	Estimated Control Mortality (%)	LC50 (% of FR)	95% Fiducial Limits Upper / Lower	LC90 (% of FR)	95% Fiducial Limits Upper / Lower	Testing the Goodness of Fit - F-value	d.f.	Sig of Goodness of Fit
Cyantraniliprole (Exirel)											
	24	634	1.38 ± 0.222	9.211	5.45	3.51, 7.61	26.67	17.8, 51.88	68.893	(2, 51)	<0.001
	48	634	1.51 ± 0.243	12.194	4.80	3.12, 6.6	20.56	14.19, 37.42	77.307	(2, 51)	<0.001
	72	634	1.39 ± 0.227	16.505	4.71	2.9, 6.66	22.89	15.47, 43.13	72.491	(2, 51)	<0.001
	96	634	1.33 ± 0.223	19.180	4.46	2.61, 6.46	23.26	15.41, 45.49	67.874	(2, 51)	<0.001
	168	634	1.58 ± 0.271	24.006	3.91	2.4, 5.44	15.76	11.0, 28.27	81.401	(2, 51)	<0.001
Lambda-cyhalothrin (Hallmark)											
	24	571	1.98 ± 0.319	23.964	7.89	5.55, 10.05	23.98	18.46, 36.24	52.782	(2, 45)	<0.001
	48	571	1.9 ± 0.328	28.446	7.75	5.13, 10.16	24.66	18.53, 39.21	49.249	(2, 45)	<0.001
	72	571	1.9 ± 0.336	33.979	8.17	5.27, 10.84	26.00	19.36, 41.94	55.234	(2, 45)	<0.001
	96	571	1.86 ± 0.342	36.306	8.19	5.09, 11.05	26.68	19.57, 44.47	54.326	(2, 45)	<0.001
	168	571	1.82 ± 0.328	38.644	8.21	5.13, 11.08	27.41	20.14, 45.3	60.458	(2, 45)	<0.001
Pyrethrum (Pyrethrum 5C)											
	24	399	1.43 ± 0.413	6.508	2.21	0.84, 3.49	10.28	5.95, 54.4	11.668	(2, 33)	<0.001
	48	399	1.41 ± 0.409	8.169	2.22	0.82, 3.53	10.50	6.04, 56.27	11.539	(2, 33)	<0.001
	72	399	1.34 ± 0.399	8.225	2.03	0.65, 3.29	10.42	5.9, 63.15	10.592	(2, 33)	<0.001
	96	399	1.35 ± 0.404	9.832	2.04	0.63, 3.35	10.45	5.89, 65.15	10.437	(2, 33)	<0.001
	168	399	1.34 ± 0.412	13.156	2.11	0.59, 3.54	10.84	5.99, 75.82	10.151	(2, 33)	<0.001

Spinosad (Tracer)										
24	414	1.49 ± 0.378	5.920	15.52	7.71, 24.12	67.70	39.76, 259.28	15.219	(2, 33)	<0.001
48	414	1.48 ± 0.423	6.061	13.68	5.61, 22.37	60.08	33.92, 318.67	12.248	(2, 33)	<0.001
72	414	1.51 ± 0.433	6.143	13.07	5.35, 21.38	55.91	31.7, 293.11	12.188	(2, 33)	<0.001
96	414	1.5 ± 0.422	6.233	12.72	5.27, 20.7	55.22	31.43, 275.55	12.320	(2, 33)	<0.001
168	414	1.54 ± 0.434	7.857	11.35	4.58, 18.45	47.41	27.35, 220.29	12.329	(2, 33)	<0.001

198

199

200 1.4.2 Rate response: mortality

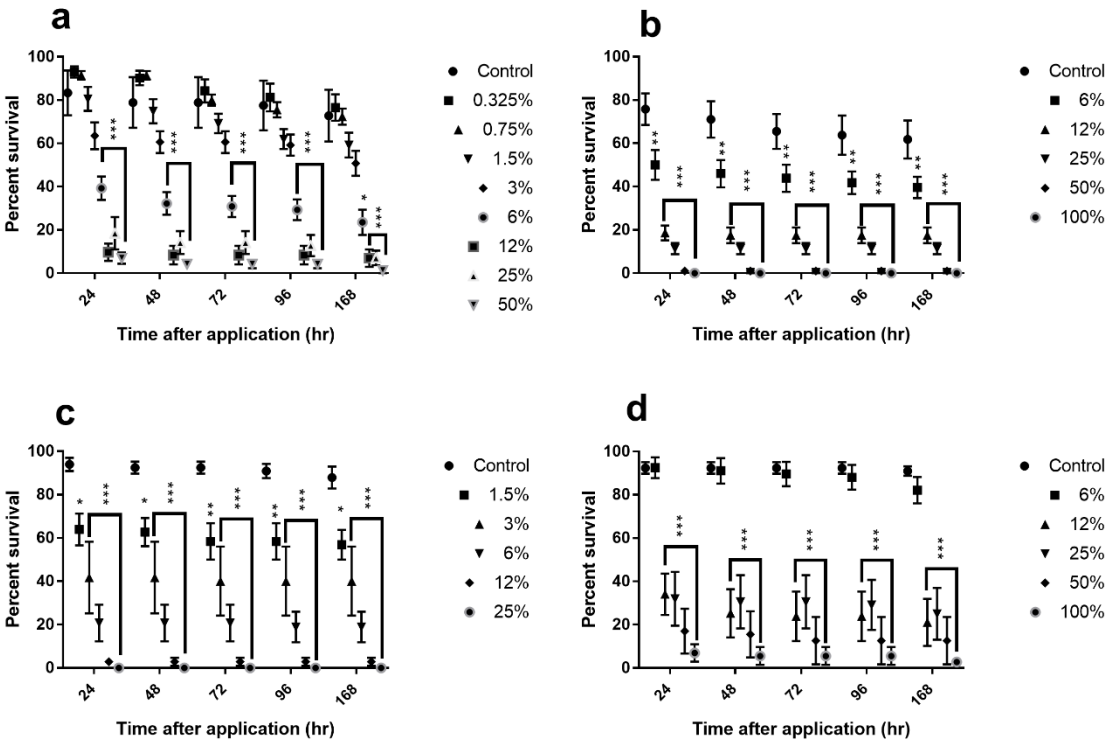
201 Survival was reduced in flies treated with 12-50% FR of cyantraniliprole in comparison to the control
202 24-168 hr after application (Figure 1a). Survival was also reduced in flies treated with 6% of
203 cyantraniliprole 24-96 hr after application, but there was no difference at 168. Twenty-four hr after
204 spray application, only 7% survival of flies treated with 50% FR resulted; this gradually declined to
205 1% after 168 hr. No difference in mortality was detected in flies treated with 0.3-3% FR of
206 cyantraniliprole in comparison to the control on any assessment day after treatment application.

207 There was a significant reduction in survival between the control and all treatment 24-168 hr after
208 application with lambda-cyhalothrin (Figure 1b). After 24 hr, there was no survival of *D. suzukii*
209 treated with 100% FR and only 1% survival in flies treated with 50% FR.

210 For pyrethrum, there was a significant reduction in survival rates for all rates from 24-168 hr after
211 application in comparison to the control (Figure 1c). No survival occurred after 24 hr in 25% FR, the
212 highest rate applied.

213 There was no significant difference in survival between the control and flies treated with 6% FR of
214 spinosad from 24-168 hr after application (Figure 1d). Twenty-four hr after treatment application,
215 an average of 7% *D. suzukii* survived 100% FR for spinosad, which fell to 3% 168 hr after application.

216 There was no statistical difference in survival over time in any of the rates of any of the insecticides
217 indicating that there was no delayed impact on mortality.



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219 Figure 1. Survival rate response of *D. suzukii* adults to dilutions of field rate (a) cyantraniliprole, (b)
220 lambda-cyhalothrin, (c) pyrethrum and (d) spinosad over time after application. * denotes
221 significant difference between the control and treatments. * $p<0.05$, ** $p<0.01$, ***$p<0.001$.
222 Bracket encompasses those points with same level of significant difference to the control.

223

224 1.4.3 Rate response: oviposition

225 Significantly more eggs were laid in the control than by adult flies treated with 12-50% FR
226 cyantraniliprole, 24-168 hr after application and more eggs were laid in the control than 3 and 6%
227 FR 24-72 hr after application (Figure 2a; Table 1 in Appendix A). There was no difference in the total
228 numbers of eggs laid in the control, 0.75 and 1.5% FR from at any time after application. More eggs
229 were laid, in total in 0.325% FR treated flies than the control 24, 96 and 168 hr after application.

230 Oviposition per female was also influenced by cyantraniliprole concentration (Figure 2a right, Table
231 1 in Appendix A). Twenty-four hr after application, rates between 3-50% FR reduced the numbers
232 of eggs per live female (EPF) in comparison to the control. Forty-eight hr after application, a

reduction in EPF in 12 and 25% FR occurred in comparison to the control. Seventy two hr after application, a reduction in EPF compared to the control was detected for 3, 6 and 25% FR. 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 hr after application and an increase occurred in EPF when treated with 0.325% FR 24 hr after application in comparison to the control. From 96 hr onwards, there was no difference between the control and any of the rates in EPF.

For lambda-cyhalothrin, significantly fewer eggs were laid from 24-168 hr after application in treatments which were 12% FR and above in comparison to the control (Figure 2b left, Table 2 in Appendix A). Fewer eggs were also laid in the 6% FR treatment 24, 72 and 96 hr after application. No eggs were laid in the 100% FR from 48 hr onwards due to 100% mortality in parent flies at the 24 hr assessment. A significant reduction in the number of EPF laid 24 hr after application occurred in all treatments in comparison to the control (Figure 2b right, Table 2 in Appendix A). There was no significant difference in the EPF between the control and 6-50% FR from 48 hr onwards.

Significantly fewer eggs were laid, in total, in 6-25% FR pyrethrum from 24-168 hr after application in comparison to the control (Figure 2c left, Table 3 in Appendix A). There was also a significant reduction in total number of eggs in the 3% FR treatment in comparison to the control from 48-96 hr after application. No oviposition occurred in 25% FR treatment from 48 hr onwards due to 100% mortality of parent flies at the 24 hr assessment. Significantly fewer EPF were laid in 6-25% FR pyrethrum treated females, in comparison to the control, 24 hr after application but no difference occurred between the control and any other treatments 48 hr onwards (Figure 2c right, Table 3 in Appendix A).

The total numbers of eggs was significantly lower compared to the control in *D. suzukii* treated with 25-100% FR spinosad from 24 hr and in *D. suzukii* treated with 12-100% FR from 48-168 hr after application. At 48 and 168 hr after application with 6% FR, there was a reduction in comparison to the control (Figure 2d left, Table 4 in Appendix A). Twenty-four hr 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 any rates at 48, 72 and 96 hr after application occurred (Figure 2d right, Table 4 in Appendix A). At 168 hr after application, there was a significant difference between the control and 6% with fewer eggs per female in those treated with 6%.

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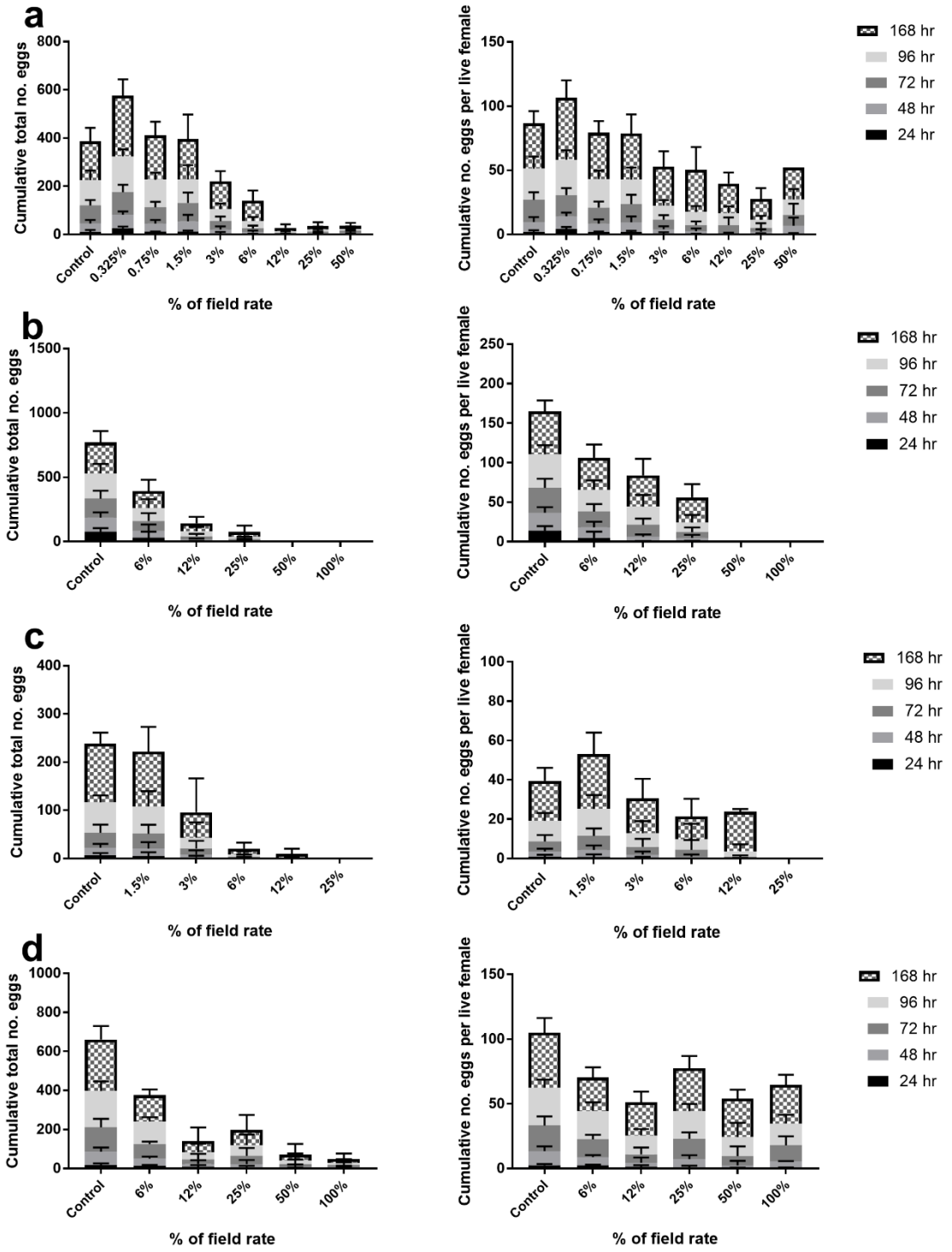


265

266 Figure 2. Mean cumulative number of total eggs (+SD) (left) and cumulative eggs per live *D. suzukii*
267 female (+SD) (right) over time after application (hr) treated with dilutions of (a) cyantraniliprole, (b)
268 lambda-cyhalothrin, (c) pyrethrum and (d) spinosad field rate.

1.4.4 Rate response: transgenerational impacts on survival

For cyantraniliprole and pyrethrum, no significant reduction in survival of eggs through to pupae formation or from pupa to adult emergence occurred at any of the rates in comparison to the distilled water control at any time point.

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

For offspring from eggs laid 48 hr after parental treatment, pupa to adult emergence was reduced ($F_{3,20}$=4.53, p=0.014) within the 6% FR (t_{20}=2.14, p<0.045) compared to the distilled water control (Figure 3).

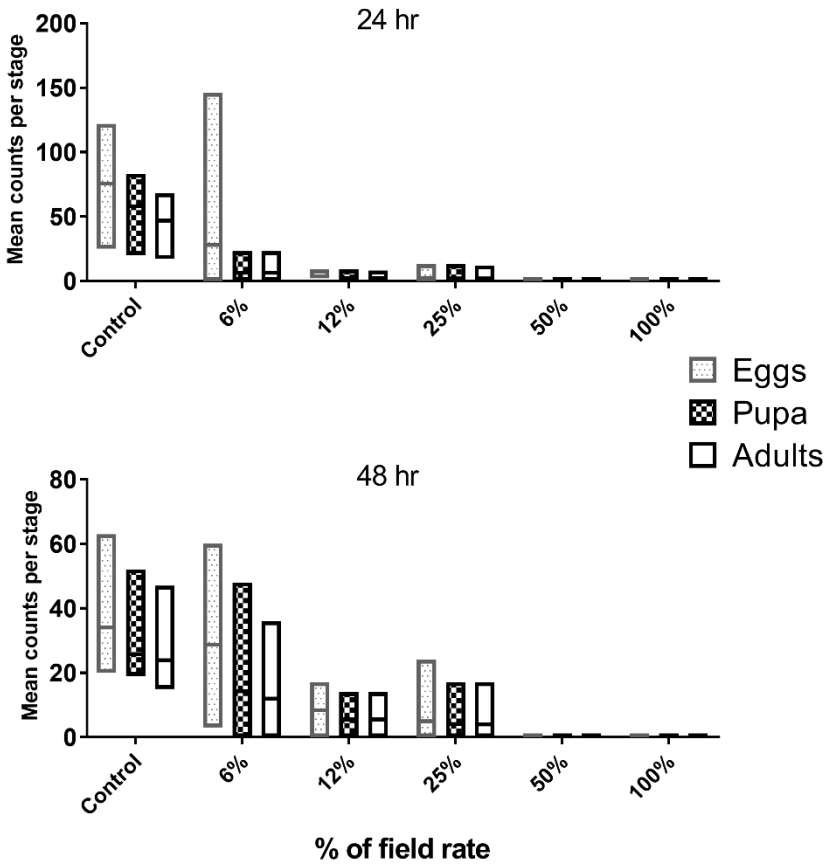
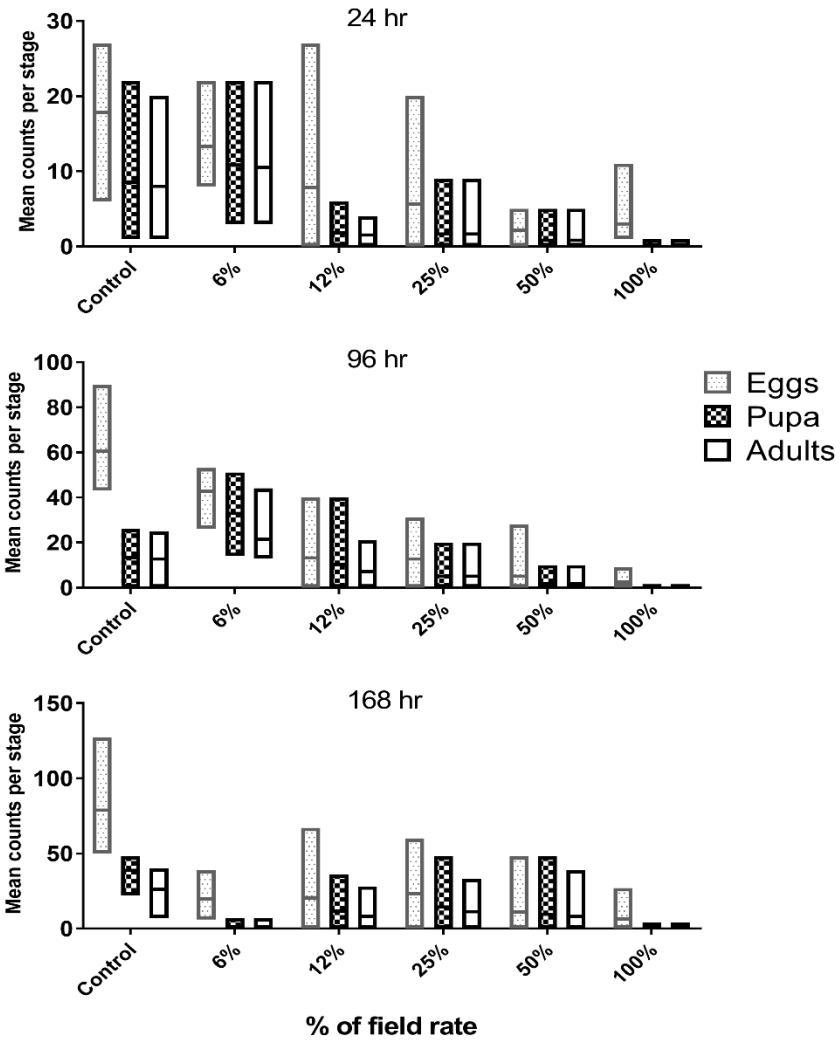


Figure 3. Mean (+SD) survival of *D. suzukii* offspring life stages from eggs collected 24 hr and 48 hr after parents were treated with lambda-cyhalothrin dilutions of field rate (75ml/ha).'Eggs' refers to the number of eggs observed at the start of incubation. 'Pupa' and 'Adults' refers to the number of formed pupa or emerged adult offspring 14 and 21 days after the start of incubation respectively.

For spinosad, a reduction in development from eggs to pupa ($F_{5,26}$=3.24, p=0.021), and egg to adult survival ($F_{5,26}$=3.75, p=0.011) in offspring from eggs laid 24 hr after parental treatment was observed. Egg to pupa (t_{26}=2.16, p=0.04) and egg to adult (t_{26}=2.34, p=0.027) survival was reduced at 6% FR compared to the control (Figure 4).

There was a significant difference in egg to pupa development for offspring from eggs laid 96 hr after application ($F_{5,16}$=3.46, p=0.026) with a reduction in egg to pupa survival in 6 and 12% FR

296 (t_{16}=3.34, p=0.004 and t_{16}=2.24, p=0.033 respectively) (Figure 4). Egg to adult survival was also
297 reduced 168 hr after application ($F_{5,16}$=3.11, p=0.038) (Figure 4) at 100% FR compared to the
298 control.



299

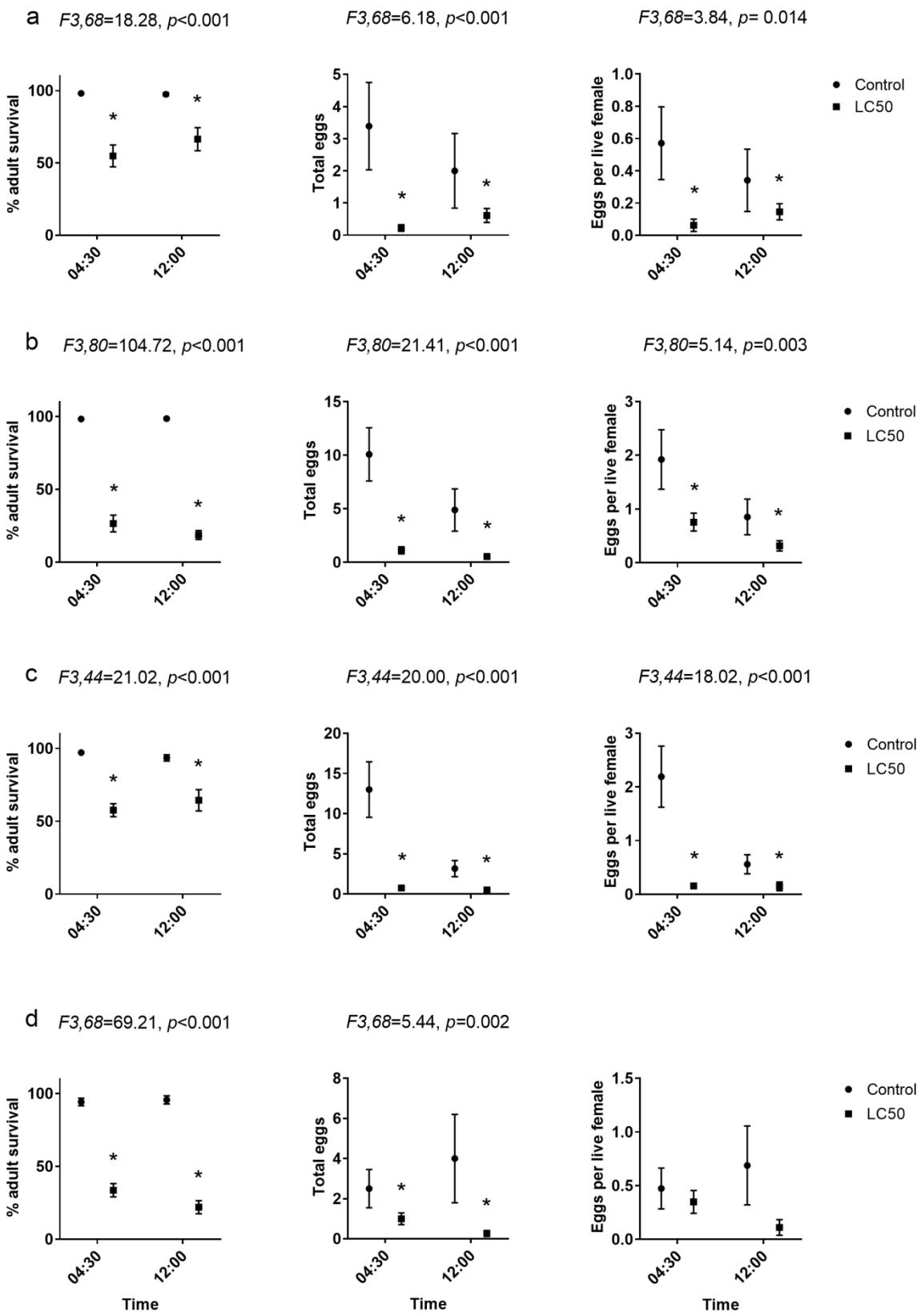
300 Figure 4. Mean (+SD) survival of *D. suzukii* offspring life stages from eggs collected 24, 96 and 168
301 hr after parents were treated with spinosad dilutions of field rate (150ml/ha). 'Eggs' refers to the
302 number of eggs observed at the start of incubation. 'Pupa' and 'Adults' refers to the number of
303 formed pupa or emerged adult offspring 14 and 21 days after the start of incubation respectively.

304

1.4.5 Chronotoxicity

Both survival and the total number of *D. suzukii* eggs laid in each insecticide and time combination (04:30 and 12:00) was reduced in comparison to the control (Figure 5). 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 (see Figure 5 for statistics), however, no difference was observed in the number of eggs laid per female between the control and *D. suzukii* treated with the LC50 rate of spinosad.

No significant difference was identified in mortality, total eggs or eggs per female between LC50 treated *D. suzukii* in relation to time for any of the insecticides. Hence, in this study, chronotoxicity had no effect on *D. suzukii* adult mortality or oviposition.



315

316 Figure 5.Effects of chronotoxicity on; *D. suzukii* adult mortality (left), total number of eggs (middle)
317 and number of eggs per treated female (right) at the LC50 of (a) cyantraniliprole, (b) lambda-
318 cyhalothrin, (c) pyrethrum and (d) spinosad 24 hr after treatment application.

1.5 Discussion

In this study, we were unable to detect any impact of chronotoxicity on mortality or oviposition rates of *D. suzukii* to exposure to pyrethrum, lambda cyhalothrin, cyantraniliprole or spinosad between two daily time points (04:30 and 12:00). However, we did identify previously unobserved interactions with sub-lethal rates of insecticide on adult mortality, oviposition and the survival of immature stages. As chemical applications play a large role in controlling *D. suzukii* in fruit crops (Andreazza et al., 2018, Beers et al., 2011, Bruck et al., 2011, Cowles et al., 2015, Pavlova et al., 2017), increasing our knowledge on how the pest interacts with insecticides is of vital importance. Both positive and negative impacts of sub-lethal rates were observed, which are likely to have subsequent consequences for both population growth and insect resistance management of *D. suzukii*.

The application of lethal rates of insecticides to crops is a legal requirement (IRAC), however, it is not uncommon for pests to come into contact with sub-lethal rates (Coats, 1991) as insecticides degrade after application due to environmental factors including light, temperature, rainfall and humidity (Fenner et al., 2013). The impact that sub-lethal rates had on mortality in this study varied greatly between the insecticides applied. For example, adult *D. suzukii* 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. Due to the high mortality at the lowest rates of pyrethrum and lambda-cyhalothrin, additional rates were added to these experiments as a range of effective and ineffective rates are required in order for the Probit analysis to calculate the LC50 rate (Van Timmeren et al., 2018).

There was no difference in mortality over time for flies treated with any rate of the insecticides tested. Hence, if they survived the first 24 hr after exposure, they survived for at least 168 hr. In

most cases there was a reduction in total eggs laid when adults were exposed to the range of rates of each insecticide. This reduction was primarily the result of increased mortality in adults. Consequently, the number of eggs per live female was calculated to distinguish if there was any impact of rate 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 (33.7-562.5 ml/ha) 24 hr after application. However, at 48 hr there was no difference between 0.325-6 and 50% (3.7-67.5 and 562.5 ml/ha) treated *D. suzukii* and the control, and at 96 hr there was no difference between any of the treated *D. suzukii* and the control (3.7-562.5 ml/ha). This trend could be interpreted as the flies recovering from the spray treatment overtime. By the end of the assessment period, all surviving females treated with all rates were laying the same number of eggs as the untreated control. Hence, if adult *D. suzukii* survives an initial spray of cyantraniliprole sub-lethal rates, there were no lasting negative effects on oviposition. This trend was also observed 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 hr onwards in all rates. 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 (9 ml/ha) 168 hr after exposure. However, this was not the case in *D. suzukii* treated with 12-100% FR (18-150 ml/ha) spinosad, in which, by 168 hr females were laying as many eggs per female as those in the untreated control.

We also observed that more eggs were laid, in total, and a higher number of eggs per female were laid by flies treated with 0.325% FR (3.7 ml/ha) cyantraniliprole; the only incidence of an increase in oviposition observed within this study. This indicates that the application of the lower rate, promoted an increase of egg production. This has also been reported in other insects, in which a positive response occurs after an application of insecticide (Calabrese and Mattson, 2011). The beneficial impact of sub-lethal rates on an organism, known as hormesis (Müller, 2018),can have a positive effect on reproductive success in pest species (Cutler, 2013). These benefits have been

reported as improving sex pheromone responses in some Lepidoptera leading to increasing mating success (Lalouette et al., 2016, Rabhi et al., 2014) and increasing reproductive rate in aphid species (Ayyanath et al., 2013, Cutler et al., 2009, Wang et al., 2017). Hence the exposure of *D. suzukii* to sub-lethal insecticide rates (dependent on active ingredient), either directly or indirectly via residues that degrade on foliage over time (Fenner et al., 2013), could increase reproductive output.

The majority of sub-lethal and lethal rates did not reduce *D. suzukii* survival or decrease the numbers of eggs laid per female. However, there were repercussions on immature stages. Not all eggs laid within these assay developed through to maturity, and the impact of treating parent flies with lowered insecticide rates had a negative effect on offspring survival. For both lambda-cyhalothrin and spinosad, there were reductions in egg survival at some rates 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, Szabo and Bakonyl, 2017). In our assay, possibly the most concerning finding was no difference in the number of eggs produced by an individual female 48 hr after spray application with 100% FR spinosad (150 ml/ha) compared to the control. However, there was a reduction in the survival of eggs through to pupal development and adult emergence at 96 and 168 hr after spray application, 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. However, transgenerational impacts can disrupt the life cycle of an insect, such as increasing generational time. When mustard leaf beetle, *Phaedon cochleariae* (Fabricius), parents are treated with sub-lethal rates insecticides, the period spent as larvae is prolonged in the offspring (Muller et al., 2017). While this does not impact overall offspring survival, it does increase larval predation opportunities (Häggström and Larsson, 1995). We did not study generation time, and so, it is not known if sub-lethal rates prolong development in *D. suzukii.* Although, if there had been an increase in generational time, we would have detected more

significant differences in the eggs to pupae and pupae to adult numbers due to the specific time points that numbers of pupae and adults were counted (14 and 21 days respectively). There may have been transgenerational effects in flies treated with cyantraniliprole or pyrethrum; however there was no impact on overall offspring survival.

As there was no reduction in eggs reaching maturity, an overall population increase occurred in flies treated with 0.325% FR (3.7ml/ha) cyantraniliprole. Although we only observed the first generation, transgenerational hormesis has been identified in aphid; 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 rates of insecticides, there is an opportunity for resistance 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 (Brevik et al., 2018, Guedes et al., 2017). 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 insecticides (Wiman et al., 2016).

To determine whether resistance was occurring within field populations, Van Timmeren et al. (2018) evaluated the susceptibility of *D. suzukii* to four insecticides over a three year period in blueberry crops. Although they found very little difference in methomyl and zeta-cypermethrin sensitivity, they did find a 'steady increase' in malathion and spinetoram tolerance over the three years. Having a baseline of susceptibility identified now means comparisons can be made in the future to monitor any possible resistance build-up. These measures can also be made to compare resistance between different regional locations.

Smirle et al. (2017) also investigated the rate response of *D. suzukii* populations established from wild strains to 11 insecticides, to identify baseline susceptibility for future comparisons. They identified the LC50 value with 3 of the 4 insecticides we studied here, cyantraniliprole, lambda-cyhalothrin and spinosad. However, they assessed mortality to residues and not direct application and we are, therefore, unable to make direct comparisons between LC50 values. In addition, in repeat exposure assays they found no change in susceptibility to malathion over 30 generations.

The initial aim of this assay was to examine the effect of the daily phase of insecticide application on *D. suzukii* susceptibility to four commonly used products. Chronotoxicity has been previously identified in *D. suzukii* to malathion (Hamby et al., 2013) and has been extensively investigated in *D. melanogaster* (Beaver et al., 2010, Misra et al., 2011, Willoughby et al., 2006, Yang et al., 2007). However, we were unable to confirm any impact of chronotoxicity of pyrethrum, lambda-cyhalothrin, cyantraniliprole or spinosad between the time points, 04:30 and 12:00, which coincided with the trough and peak of temperature and *D. suzukii* activity. Shipp and Otton (1976) detected chronotoxicity in *M. domestica* to the three insecticides applied within their assay with susceptibility to DDT, dieldrin and malathion peaking 1 h before 'sun-rise'. Hamby et al. (2013) found chronotoxic effects to one of the two insecticides assayed. Sensitivity to malathion did vary between application times indicated by rise or fall in mortality levels. However, there was no impact of time of application on fenpropathrin susceptibility. Fenpropathrin is a synthetic pyrethroid, similar to lambda-cyhalothrin. In our study we found no impact of time on susceptibility to lambda-cyhalothrin. Both fenpropathrin and lambda-cyhalothrin along with pyrethrum, which was also evaluated within our assay, are sodium channel modulators (Qiu et al., 2007, Sparks and Nauen, 2015). Spinosad targets nicotinic acetylcholine receptors (Salgado, 1998, Sparks and Nauen, 2015) whilst malathion targets acetylcholinesterase (Can, 2014). It is likely that chronotoxic effects are not implicated in *Drosophila* to all insecticides or that specific classes or modes of action exhibit this effect. In addition we investigated only 2 time points and could have missed detecting chronotoxic effects.

The data presented here increase the knowledge of sub-lethal rates of insecticides on the mortality of parent flies, oviposition and transgenerational effects on offspring survival in *D. suzukii*. Perry et al. (2011) stress the importance of having a greater knowledge of the biology of an insect when proceeding with insecticide resistance investigations and this should be factored into methodologies in future research. The sub-lethal impacts of reduced insecticide rate have revealed differences between insecticide groups in their toxicological effects and the importance of rotation and resistance management strategies for *D. suzukii*.

1.6 Conclusion

We were unable to detect a variation in susceptibility of *D. suzukii* to cyantraniliprole, lambda-cyhalothrin, pyrethrum and spinosad in relation to time of application in a 24 h period. There were a range of responses to rate including oviposition and offspring survival interactions. Future work should focus on strains established from recent wild populations from a range of low to high insecticide inputs to establish whether resistance is beginning to occur in field populations. Chronotoxicity should be pursued but testing additional timings in relation to the circadian clock. Further transgenerational impacts such as development time and subsequent offspring impacts should also be explored. By increasing the knowledge surrounding how *D. suzukii* interacts with chemical control, we are able to not only improve best practice techniques and the guidance provided to fruit growers but also to mitigate insecticide resistance development. As there are limited modes of action available to control *D. suzukii*, it is necessary to understand how these impact the genetic, organism and population levels to inform decisions on timing, frequency and rotation of use in the field.

467 Acknowledgments

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472

1.7 Appendix

Appendix A.

Table 1. Overall significance and pairwise interactions of (top) total number of eggs and (bottom) number of *D. suzukii* eggs per female, in each rate treated with cyantraniliprole. Results are provided for overall and pairwise comparisons to a distilled water control, over time, after application. -indicates no significant difference.* Overall significance produced by significant differences between 0.325 and 1.5, 3 and 6% and 0.75 and 3%.

Total eggs	**Time (h after application)**									
	24		**48**		**72**		**96**		**168**	
Regression, residual	8,45		8,45		8,45		8,45		8,45	
***F* value**	10.43		14.58		15.28		12.79		9.75	
***p* value**	<0.001		<0.001		<0.001		<0.001		<0.001	
	pairwise comparisons with control									
Rate (%FR)	*p* value	*F* value	*p* value	*F* value	*p* value	*F* value	*p* value	*F* value	*p* value	*F* value
0.325	0.002	10.45	-	-	-	-	0.035	4.71	0.036	4.69
0.75	-	-	-	-	-	-	-	-	-	-
1.5	-	-	-	-	-	-	-	-	-	-
3	0.032	4.87	0.044	4.28	0.009	7.38	-	-	-	-
6	0.002	10.63	0.001	13.50	<0.001	21.55	-	-	-	-
12	0.002	11.35	<0.001	36.45	<0.001	33.48	<0.001	20.97	0.001	12.34
25	0.012	6.77	<0.001	28.10	<0.001	39.37	<0.001	18.37	<0.001	14.30
50	0.012	6.77	<0.001	21.58	<0.001	43.54	<0.001	21.46	<0.001	15.25

Eggs per female	**Time (h after application)**									
	24		**48**		**72**		**96**		**168**	
Regression, residual	8,45		8,36		8,36		8,36		-	
***F* value**	10		2.39		3.26		2.4		-	
***p* value**	<0.001		0.036		0.007		0.035*		-	
	pairwise comparisons with control									
Rate (%FR)	*p* value	*F* value	*p* value	*F* value	*p* value	*F* value	*p* value	*F* value	*p* value	*F* value
0.325	0.004	9.26	-	-	-		-	-	-	-
0.75	-	-	-	-	-	-	-	-	-	-
1.5	-	-	-	-	-	-	-	-	-	-
3	0.033	4.85	-	-	0.008	7.83	-	-	-	-
6	0.003	10.01	-	-	0.001	13.78	-	-	-	-
12	0.002	11.03	0.034	4.85	-	-	-	-	-	-
25	0.010	7.24	0.050	4.10	0.004	9.29	-	-	-	-
50	0.011	6.96	-	-	-	-	-	-	-	-

480

481 Table 2. Overall significance and pairwise interactions of (top) total number of eggs and (bottom)
482 number of *D. suzukii* eggs per female, in each rate treated with lambda-cyhalothrin. Results are
483 provided for overall and pairwise comparisons to a distilled water control, over time, after
484 application. - indicates no significant difference.

Total eggs	**Time (h after application)**									
	24		**48**		**72**		**96**		**168**	
Regression, residual	5,42		5,43		5,44		5,45		5,46	
***F* value**	25.96		22.40		15.97		22.55		14.72	
***p* value**	<0.001		<0.001		<0.001		<0.001		<0.001	
Rate (%FR)	**pairwise comparisons with control**									
	p value	*F* value	*p* value	*F* value	*p* value	*F* value	*p* value	*F* value	*p* value	*F* value
6	<0.000	13.71	-	-	0.025	5.38	0.005	8.81	-	-
12	<0.001	48.27	<0.001	19.12	0.003	9.87	<0.001	20.89	0.020	5.86
25	<0.001	52.57	<0.001	27.80	<0.001	27.55	<0.001	34.07	0.007	8.01
50	<0.001	62.94	<0.001	54.05	<0.001	43.69	<0.001	65.13	<0.001	41.52
100	<0.001	61.94	<0.001	54.05	<0.001	44.97	<0.001	65.13	<0.001	42.52

Eggs per female	**Time (h after application)**									
	24		**48**		**72**		**96**		**168**	
Regression, residual	5,42		-		-		-		-	
***F* value**	26.43		-		-		-		-	
***p* value**	>0.001		-		-		-		-	
Rate (%FR)	**pairwise comparisons with control**									
	p value	*F* value	*p* value	*F* value	*p* value	*F* value	*p* value	*F* value	*p* value	*F* value
6	<0.001	15.78	-	-	-	-	-	-	-	-
12	<0.001	52.22	-	-	-	-	-	-	-	-
25	<0.001	54.64	-	-	-	-	-	-	-	-
50	<0.001	64.79	-	-	-	-	-	-	-	-
100	<0.001	63.81								

485

486

Table 3. Overall significance and pairwise interactions of (top) total number of eggs and (bottom) number of *D. suzukii* eggs per female, in each rate treated with pyrethrum. Results are provided for overall and pairwise comparisons to a distilled water control, over time, after application. - indicates no significant difference

Total eggs	**Time (h after application)**									
	24		**48**		**72**		**96**		**168**	
Regression, residual	5,30		5,30		5,30		5,30		5,30	
***F* value**	5.08		12.43		10.40		17.06		12.33	
***p* value**	<0.001		<0.001		<0.001		<0.001		<0.001	
Rate (%FR)	**pairwise comparisons with control**									
	p value	*F* value	*p* value	*F* value	*p* value	*F* value	*p* value	*F* value	*p* value	*F* value
1.5	-	-	-	-	-	-	-	-	-	-
3	-	-	0.045	4.40	0.041	4.58	0.002	11.02	-	-
6	0.001	12.46	0.001	14.06	0.001	14.22	<0.001	30.83	<0.001	18.66
12	0.005	9.30	<0.001	23.28	<0.001	22.93	<0.001	35.76	<0.001	20.41
25	0.001	12.46	<0.001	23.28	<0.001	24.79	<0.001	40.89	<0.001	33.02

Eggs per female	**Time (h after application)**									
	24		**48**		**72**		**96**		**168**	
Regression, residual	5,30		-		-		-		-	
***F* value**	4.25		-		-		-		-	
***p* value**	0.005		-		-		-		-	
Rate (%FR)	**pairwise comparisons with control**									
	p value	*F* value	*p* value	*F* value	*p* value	*F* value	*p* value	*F* value	*p* value	*F* value
1.5	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-
6	0.003	10.42	-	-	-	-	-	-	-	-
12	0.012	7.14	-	-	-	-	-	-	-	-
25	0.004	9.78								

494 Table 4. Overall significance and pairwise interactions of (top) total number of eggs and (bottom)
495 number of *D. suzukii* eggs per female, in each rate treated with spinosad. Results are provided for
496 overall and pairwise comparisons to a distilled water control, over time, after application. - indicates
497 no significant difference

Total eggs	**Time (h after application)**									
	24		**48**		**72**		**96**		**168**	
Regression, residual	5,30		5,30		5,30		5,30		5,30	
***F* value**	4.53		8.08		6.59		10.64		4.93	
***p* value**	<0.001		<0.001		<0.001		<0.001		<0.001	
Rate (%FR)	**pairwise comparisons with control**									
	p value	*F* value	*p* value	*F* value	*p* value	*F* value	*p* value	*F* value	*p* value	*F* value
6	-	-	0.040	4.60	-	-	-	-	0.006	8.93
12	-	-	<0.001	16.29	0.001	14.61	0.001	14.94	0.006	8.64
25	0.015	6.61	0.001	12.23	0.041	4.58	<0.001	15.41	0.010	7.53
50	0.001	13.98	<0.001	21.06	<0.001	15.64	<0.001	25.14	0.001	13.65
100	0.002	11.69	<0.001	25.26	<0.001	18.88	<0.001	30.44	<0.001	17.10

Eggs per female	**Time (h after application)**									
	24		**48**		**72**		**96**		**168**	
Regression, residual	5,30		-		-		-		5,16	
***F* value**	4.88		-		-		-		3.15	
***p* value**	0.002		-		-		-		0.036	
Rate (%FR)	.									
	p value	*F* value	*p* value	*F* value	*p* value	*F* value	*p* value	*F* value	*p* value	*F* value
6	-	-	-	-	-	-	-	-	0.002	14.0
12	0.045	4.39	-	-	-	-	-	-	-	-
25	0.018	6.28	-	-	-	-	-	-	-	-
50	0.001	13.66	-	-	-	-	-	-	-	-
100	0.002	12.04	-	-	-	-	-	-	-	-

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