Title: Reducing *Drosophila suzukii* emergence through inter-species competition

Running title: Reducing *D. suzukii* through competition

Authors: *Bethan Shaw [1], [2], Philip Brain[1], Herman Wijnen [2], Michelle T Fountain [1]*

[1]NIAB EMR, East Malling Kent, ME19 6BJ, UK

[2] University of Southampton, Southampton, UK

Contact details:

Bethan Shaw (corresponding author). Email: bethan.shaw@emr.ac.uk. Tel: +44 (0) 1732 843833. Fax: +44 (0) 1732 849067

Philip Brain. Email: philip.brain@emr.ac.uk. Tel: +44 (0) 1732 843833. Fax: +44 (0) 1732 849067

Herman Wijnen. Email: h.wijnen@soton.ac.uk. Tel: + (0) 44 23 8059 4336. Fax: +44 (0) 23 8059 3131

Michelle T Fountain. Email: michelle.fountain@emr.ac.uk. Tel: +44 (0) 1732 843833. Fax: +44 (0) 1732 849067

Abstract

BACKGROUND: *Drosophila suzukii* has dispersed widely from its native Asian range since 2008. Its arrival in the UK is resulting in economic losses in soft- and stone-fruit crops caused by larvae feeding on the flesh of ripening fruit. Although a large amount of research has been directed at controlling this pest, it is presently unknown how this invasive species interacts with native *Drosophila* species.

RESULTS: In the work reported here *Drosophila suzukii* or *Drosophila melanogaster* adults were introduced to substrates, pre-inoculated with the eggs of the same or other species in a laboratory choice assay. *D. melanogaster* adult emergence was not affected by the pre-inoculation of *D. suzukii*. *D. suzukii* was significantly lower from media pre-inoculated by *D. melanogaster* than from blank media. In a following experiment, significantly more *D. suzukii* eggs were laid in blank media than in *D. melanogaster* pre-inoculated media.

CONCLUSION: The presence of *D. melanogaster* in a substrate significantly reduced *D. suzukii* emergence and egg laying. This study opens future research questions on how this reduction mechanism is driven and how it could be exploited as part of future Integrated Pest Management practices.

Keywords: *Drosophila melanogaster*; fruit; IPM, oviposition; repellents;

Headings:

1 INTRODUCTION

Drosophila suzukii Matsumura (*D. suzukii*) is a relatively new invasive pest of soft-, stone- and ornamental-fruit worldwide. Originally from Asia (1), this dipteran has dispersed globally since 2008 spreading across the Americas and Europe (2-5) aided by imported, infested fruit containing eggs and larvae (6). The females' serrated oviscapts (7, 8) cuts into the skin of healthy soft fruits to deposit eggs under the fruit epicarp. Once laid, the egg hatches and larval instars consume the fruit from within causing it to collapse (7, 8). The presence of the pest in fruit, even at low levels, can make it unmarketable.

The need to control *D. suzukii* populations has caused changes in cropping management practices, resulting in the re-establishment of routine spray programmes (9). This chemical approach disrupts Integrated Pest Management (IPM), disturbing biological control methods for other pests such as parasitoids and predatory mites (10). Although chemical control is able to reduce *D. suzukii* infestation, increasing restrictions on chemical approvals and an overall population increase of *D. suzukii* has driven research efforts into alternative methods of control (11).

Exploitative competition causes a reduction in the availability of resources and generally results in population reduction of one or both species involved. As *D. suzukii* and *D. melanogaster* occupy overlapping niches, it is realistic to presume some form of exploitative competition will occur between the two. Late in the commercial growing season it is not uncommon to find both species utilising overripe fruit for egg laying. The impact that the arrival of *D. suzukii* is having on native *Drosophila* species is currently unknown. Publications that focus on direct and indirect interactions of alien insects on native fauna and flora and competition for resources between inter- and intra-species are well documented (12). However, typically these focus on the 'socio-economically important' native species such as pollinators (including butterflies) and predators (13). Although native *Drosophila* are generally regarded as a nuisance in commercial crops they do play a vital role in most ecosystems, aiding the degradation of organic matter and providing a food source for many organisms (14). The aim of this study was to investigate whether the presence of *D. suzukii* or *D. melanogaster* would disrupt offspring emergence of the opposing species and the mechanism of this disruption.

2 EXPERIMENTAL METHODS

2.1 Drosophila cultures

77 *D. suzukii* cultures were established at NIAB EMR, Kent, UK, from an Italian strain collected in 2013. Culture
78 cages (30 x 30 x 30 cm) containing populations of *D. suzukii* were held at 25°C in a 16h:8h light/dark cycle and
79 fed a standard cornmeal diet (100 % dH_2O, 1 % Fisher agar, 9 % table sugar, 9 % precooked ground maize, 2 %
80 baker's yeast, 0.2 % methylparaben, dissolved in 10 ml 70% ethanol). Flies had unrestricted access to water and
81 were periodically provisioned with pesticide free raspberry fruit grown at NIAB EMR (mixed varieties) previously
82 frozen to eliminate naturally occurring *Drosophila*.

83 *D. melanogaster* cultures were established from a wild type culture purchased from Blades Biologicals
84 (http://www.blades-bio.co.uk/) maintained in the same environmental conditions and provided with the same
85 resources as *D. suzukii*.

86

87 **2.2 Next generation emergence experiments**

88 The next generation emergence experiments were repeated three times; once with the standard cornmeal media
89 (19/05/2015) and twice on fresh raspberry fruit (16/10/2015 and 29/07/2016).

90

91 **2.2.1 Cornmeal (Experiment 1)**

92 The standard cornmeal media (as above) was made 24 hours before commencing the trial. Petri dishes (55 mm x
93 14 mm Thermo Scientific Sterilin PF55 disposable, polyethylene, non-vented) were filled with media and left to
94 cool under a fume hood for one hour before lids were replaced. Dishes were stored in 5°C overnight and were
95 moved into 25°C one hour before commencing the experiment.

96

97 **2.2.2 Raspberry (Experiment 2 and 3)**

98 Raspberries grown at NIAB EMR were collected three days before commencing the experiment. Ripe cv.
99 'Autumn Treasure' and 'Autumn Amber' fruits were washed under distilled water for 30 seconds and then left to
100 dry on trays within the quarantine facility at NIAB EMR for 30 minutes. Fruits had received minimal insecticidal
101 sprays in both years (12/06/15 Thiacloprid, 21/07/15 Deltamethrin, 24/7/15 and 07/08/15 Abarmectin, 07/06/16
102 Pymetrozine, 21/06/16 Thiacloprid), with the final application for both years being Spinosad 04/09/15 and

22/07/16 with a harvest interval of 3 days. Once dry, the fruit was transferred to punnets and stored at 5°C for three days in order for the fruit to start degrading as *D. melanogaster* is unable to exploit intact fruit. An hour prior to starting the experiment raspberries were transferred to 55 mm Petri dishes and held at 25°C. Two raspberries, one of each variety, were allocated per dish.

2.2.3 Experimental set-up

The experiment was conducted in a contained chamber within the insect quarantine facility at NIAB EMR in the same conditions as the *Drosophila* cultures (25°C in a 16h: 8h light/dark cycle). A Petri dish of either the cornmeal media or raspberries was transferred into a 12 x 7 x 7 cm ventilated Perspex box. All *Drosophila* 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. 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 (Figure 1). 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 (Figure 1). 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 (15). 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 (16). 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.

A control sample of fruit was taken for both raspberry experiments to determine the presence of natural infestation of *D. suzukii* and was monitored for 21 days from the start of each experiment.

2.2.4 Assessments

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

2.2.5 Statistical analysis

Counts for each species, experiment and treatment (inoculated or blank substrate) were analysed separately using 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 are 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 variance between the repeat measurements on the same dish, so the "paired" nature of the design was ignored and within-, and between-, dish variances pooled. This also gave more degrees of freedom for significance testing

2.3 Female *D. suzukii* oviposition choice experiment

The *D. suzukii* oviposition choice experiment was done once (07/08/17) and followed methodology for 2.2.1 Cornmeal (experiment 1) preparation.

2.3.1 Experimental set-up

The experiment was held in the environmental conditions stated in 2.2.3. Twenty Petri dishes containing cornmeal media 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 X12 magnification. Ten Petri dishes containing between 6-20 egg cases were transferred individually into 12 x 7 x 7 cm ventilated Perspex boxes. This was to ensure media was not too densely populated initially. A new, blank Petri dish of the cornmeal media was added to each box. Boxes now contained two Petri dishes: one pre-inoculated and one with blank media. *D. suzukii* 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 10 minutes after flies had been added to ensure recovery.

2.3.2 Assessments

Forty eight hours after the second inoculation all flies were removed from the boxes and the 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 they are mobile and could have migrated from one dish to the other.

2.3.3 Statistical analysis

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

3 RESULTS

In the next generation experiments no *D. suzukii* emerged from the control fruit confirming the raspberries used did not contain a background infestation of *D. suzukii*. *D. melanogaster* and *D. suzukii* emerged from both the media and raspberry fruit experiments verifying that the fruit had degraded enough to allow *D. melanogaster* oviposition.

In all repeats of the experiment there was emergence of the first inoculated *Drosophila* species, although very low numbers of *D. suzukii* emerged from the first raspberry experiment (treatments 2 and 3) (results not shown).

Results from all three experiments are presented in Figures 2-4. Significantly more *D. suzukii* adults emerged from blank media that contained no eggs initially than media pre-inoculated with *D. melanogaster* (Figure 2) (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). There was no significant difference between the numbers of *D. melanogaster* that emerged from blank media or the *D. suzukii* pre-inoculated media (Figure 3). There was also no significant difference between the numbers of *D. suzukii* that emerged from blank media compared to media that had previously been exposed to other *D. suzukii* (Figure 4). Hence, *D. suzukii* emergence was significantly reduced by the presence of *D. melanogaster*, but *D. melanogaster* emergence was not affected by the presence of *D. suzukii*.

Significantly more *D. suzukii* eggs were counted in blank media (mean 24 eggs) than in media pre-inoculated by *D. melanogaster* (average 3 eggs) in the female oviposition choice experiment (Figure 5) ($t_9 = -3.122$; P = 0.012).

4 DISCUSSION

The original 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 significant difference in emergence from pre-inoculated and blank media. When given a choice, female *D. melanogaster* oviposited in pre-inoculated media 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 to a blank media. It is suggested that this could be due to cannibalistic tendencies of some *Drosophila* species which occurs when nutrition is restricted (17). Morphological defects can be a visual indication that cultures have been maintained on diets lacking nutrition (18). 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. However, Bhattacharyya (19, 20) found that a 'basal level of cannibalism' did occur in *D. melanogaster* cultured on a standard yeast/sugar diet, with younger larvae predating on those preparing to pupate. As *D. melanogaster* develops to the pupal stage faster than *D. suzukii*, interspecific predation would tend to cause a reduction in emergence of *D. melanogaster* and not *D. suzukii*, as found in this study. Such direct interactions between the two species are therefore unlikely to explain why *D. suzukii* emergence was lower from media pre-inoculated with *D. melanogaster*.

In our third treatment in the next generation emergence experiment, *D. suzukii* females were given a choice of blank media or media pre-inoculated with the eggs and larvae of conspecifics. There was no significant difference in emergence from these two media options. If significantly more *D. suzukii* emerged from the pre-inoculated media, it could have indicated oviposition aggregation pheromones as found in some *Drosophila* species (21, 22). However, in wild populations, if given a choice, *D. suzukii* eggs are typically deposited either singly into fruit or in very small clutches (23) indicating *D. suzukii* preference for oviposition sites low in egg counts or free from both *D. melanogaster* and conspecifics.

A recent study by Dancau et al, (24) investigated whether the direct exploitative competition of *D. melanogaster* could supress *D. suzukii* numbers at various densities in mixed culture settings over multiple generations. In pairwise, small group and cage settings they also found that the presence of *D. melanogaster* significantly reduced adult *D. suzukii* emergence in no choice experiments. However, within this study the authors do not identify the mechanism that causes this reduction stating "The mechanism may have been larval–larval interference or adult competitive exclusion of *D. suzukii* from the oviposition resource by *D. melanogaster"*.

To identify the point at which competition occurred, a female *D. suzukii* oviposition choice experiment was done. In this assay we found, on average, 23 *D. suzukii* eggs on blank media compared with an average of 3 *D. suzukii* eggs on media that had been pre-inoculated by *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 due to the *D. melanogaster* larvae predating on *D. suzukii* eggs. However, as previously discussed, cannibalism or interspecific predation primarily occurs in either low nutrient media or highly populated substrates on older immobile larvae. Vijendravarma et al, (18) found that to promote cannibalism larval density had to exceed 15 larvae per gram of standard media. In our experiments an average of 10 grams of standard media was used meaning to promote cannibalism each dish would need larval densities of 150 larvae. The highest possible density in the pre-inoculated media 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 media was at least partly due to female oviposition choice. In natural conditions, the niche that *D. suzukii* occupies means they do not normally need to compete with other *Drosophila* species for egg laying resources until ripening fruit become scarce. However, in assays performed by Bernardi et al. (25) when given a choice of varying fruit

ripeness, and with no competition, significantly more *D. suzukii* eggs were laid and adults emerged from ripe and overripe fruit compared to ripening; a trend also found by Lee *et al.* in comprehensive investigations (26, 27). There was also no significant attraction preference to under- ripe fruit volatiles in comparison to ripe or over-ripe volatiles in approach assays (28). Keesey et al. (28) discus the point that ripening fruit volatiles alone do not explain or indicate *D. suzukii's* preferences for ripening fruit as an oviposition site.

Cis-vaccenyl acetate (*c*VA) is a complex, multifunctional, male-produced pheromone used in courtship, aggression and aggregation signalling in most *Drosophila* species (29). However, *c*VA appears to have a disruptive effect on *D. suzukii*, and when applied to males, resulted in reduced mating (30). This change in pheromone perception in *D. suzukii* could be the reason females oviposit into under ripe fruit when other species have utilised overripe, detectable by the presence of *c*VA or 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* (31) and sweet potato whitefly, *Bemisia tabaci* Gennadius (32). 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 signal characteristic of infested fruit and use them as egg laying repellents.

5 CONCLUSION

Adult *D. suzukii* emergence was reduced from media pre-inoculated by *D. melanogaster* females. Also, fewer *D. suzukii* eggs were found in media pre-inoculated by *D. melanogaster*. It appears that this reduction is due to female *D. suzukii* depositing fewer eggs in these pre-inoculated substrates. *D. melanogaster* emergence was not reduced by *D. suzukii* presence and so concerns for the reduction in wild populations of this species are currently unwarranted. Further investigation into the exact point of competition is needed to understand the interactions between these two species. The potential of inter-species competition could be exploited as a control method if the mechanisms driving repellence are understood

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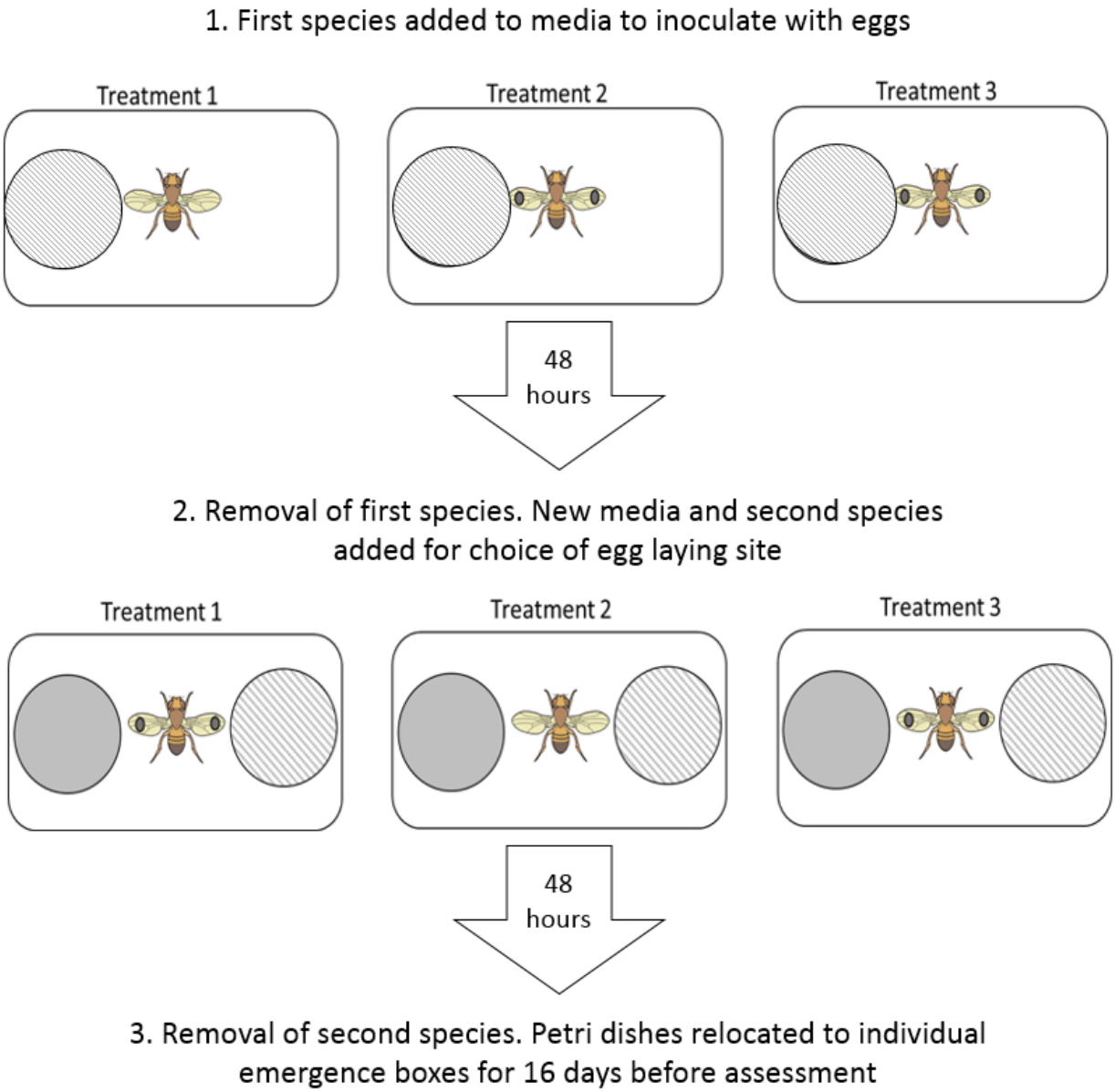

Fig. 1 Visual interpretation of successive steps of the *Drosophila* species inoculations. Fly with spots on wings indicate *D. suzukii*. Fly without spots indicates *D. melanogaster*. Dark grey disk represents substrate with eggs from first species inoculation. Light grey disks represents new, blank substrate containing no eggs initially

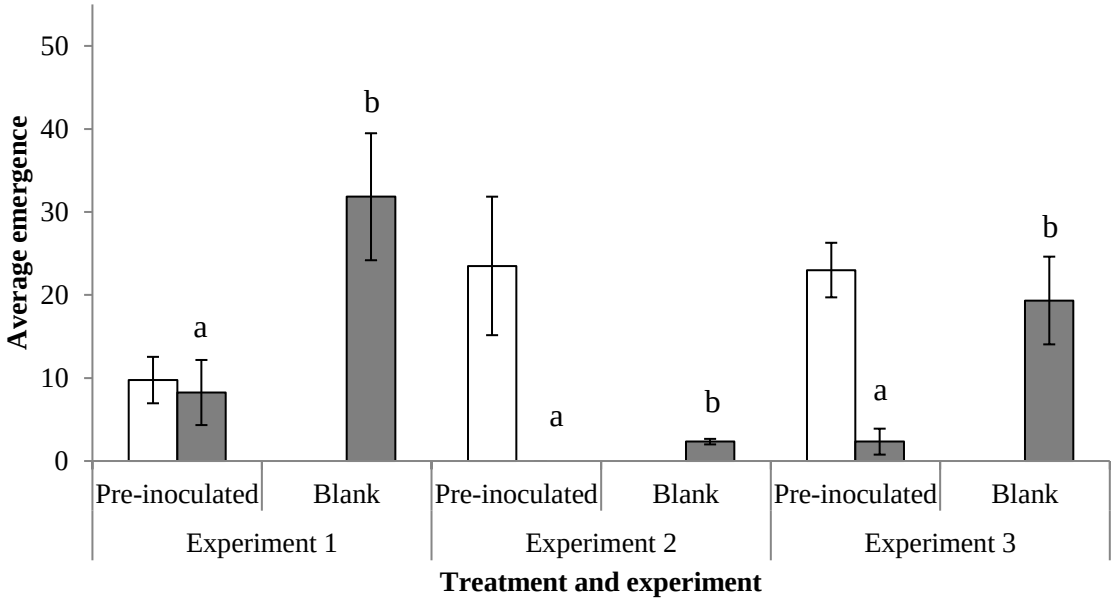

Fig. 2 Mean emergence (±S.E.) of *D. suzukii* adult offspring indicated by grey bars, from either cornmeal media (experiment 1) or raspberry fruits (experiment 2 and 3) pre-inoculated with *D. melanogaster* eggs or blank media. White bars display emergence of *D. melanogaster* pre-inoculation. Different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

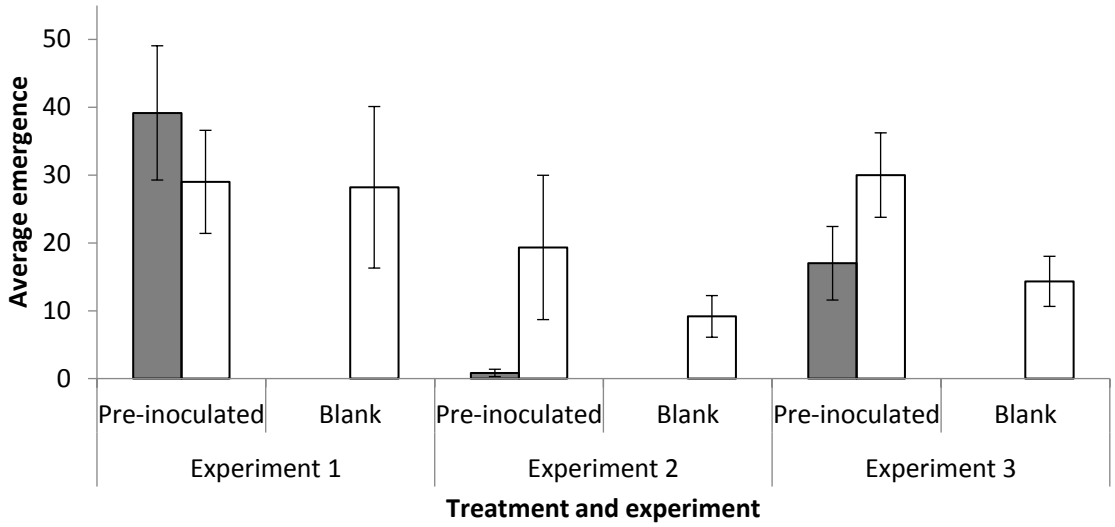

Fig. 3 Mean emergence (±S.E.) of *D. melanogaster* adult offspring indicated by white bars, from either cornmeal media (experiment 1) or raspberry fruits (experiment 2 and 3) pre-inoculated with *D. suzukii* eggs or blank media. Grey bars display emergence *D. suzukii* of pre-inoculation. NSD between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

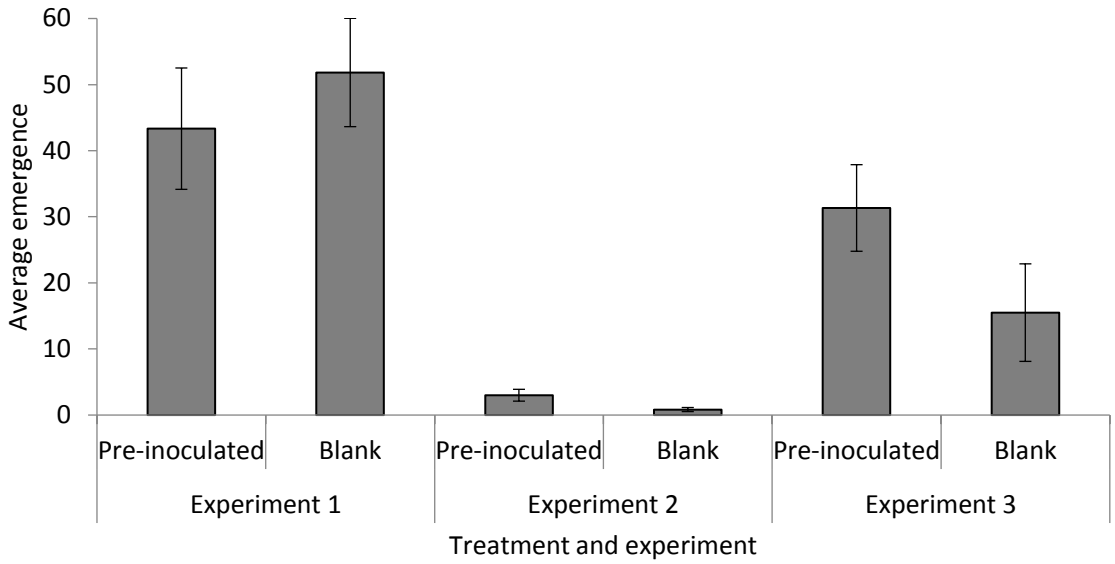

Fig. 4 Mean emergence (±S.E.) of *D. suzukii* adult offspring from either cornmeal media (experiment 1) or raspberry fruits (experiment 2 and 3) pre-inoculated with *D. suzukii* eggs or blank media. NSD between treatments in emergence from inoculated and blank media for each experiment

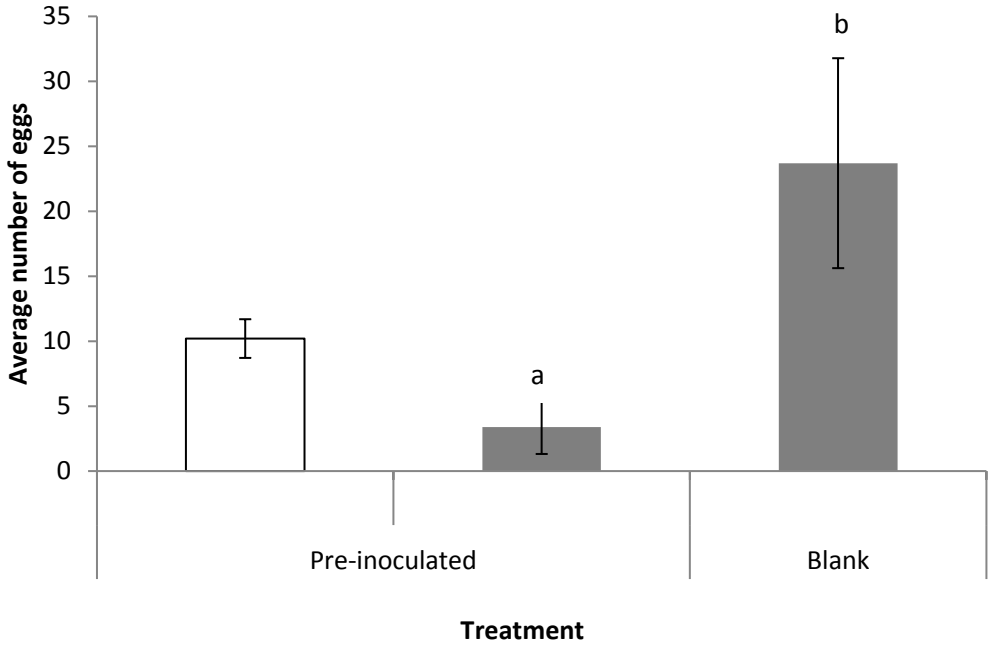

Fig. 5 Mean number (±S.E.) of *D. suzukii* eggs laid in either blank or pre-inoculated media indicated by grey bars. White bars indicate average number of *D. melanogaster* eggs in the pre-inoculation treatment.